Novel Biopesticides Based on Recombinant Avidin for Protection of Crops Against Insect Pests

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Declaration

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Abstract

Farmers are required to increase their food productivity to meet the demand from a continually enlarging population. A major constraint on meeting this requirement is the damage created by insect pests. Agricultural pests and associated diseases destroy 30%-40% of the world's crop produce. The withdrawal of broad-spectrum chemical insecticides, the threat of the development of resistance in insect pests to remaining compounds and the possibility of new pest species spreading into the UK as a result of environmental change have prompted research into alternative pesticides.

Protein-based insecticides offer the possibility of producing compounds that are specific to pest species and environmentally benign. The production of orally active insecticidal fusion proteins, containing toxins from *Segestria florentina* (tube-web spider) or *Mesobuthus tamulus* (Indian red scorpion) fused to a 'carrier' protein (snowdrop lectin; *Galanthus nivalis* agglutinin; GNA) which transports them across the insect gut epithelium, has shown that recombinant protein expression systems and protein engineering techniques can be used to produce novel insecticidal proteins. The main aim of the work described in this thesis is to extend this technology.

Avidin, a biotin-binding protein known to be insecticidal, was evaluated as a possible 'carrier' protein. Recombinant avidin was produced in high yields using *Pichia pastoris* and was compared to the native egg white protein. Recombinant avidin has insecticidal activity towards hemipteran plant pests. It was highly toxic to *Acyrthosiphon pisum* (pea aphid) when fed in liquid artificial diet, causing 100% mortality after four days when present at concentrations ≥0.25mg/ml (250ppm). The toxicity towards *A. pisum* was prevented by biotin supplementation of the diet. In contrast, recombinant avidin had no significant effects on the survival of *Sitobion avenae* (cereal aphid) at concentrations up to 2mg/ml (2000ppm) in liquid diet. Analysis of genomic DNA showed that symbionts from both aphid species lack the ability to synthesise biotin *de novo*. Cereal aphids appear to be less sensitive to sub-

optimal levels of biotin and possess a more effective system for scavenging biotin from recombinant avidin in the diet.

Avidin is readily transported to the haemolymph of lepidopteran larvae after feeding, which suggested that it might replace GNA in synthetic insecticidal fusion proteins. Numerous attempts were made to produce a fully functional insecticidal avidin-based fusion protein containing scorpion or spider toxins by expression as recombinant proteins in *P. pastoris* but, following tests against *Mamestra brassicae* (cabbage moth) larvae, the fusion proteins were found to be non-toxic. The lack of toxicity was most likely due to incorrect folding of the toxin component of the fusion, since the avidin component was functional.

Avidin fed to *A. pisum* was found to bind to the stomach region of the gut after ingestion and was retained for at least 72 hours. Feeding conjugates of avidin with fluorescently labelled biotin, or a fluorescently labelled, biotinylated peptide, showed that the conjugated compound was also retained in the aphid gut after feeding. A conjugate between avidin and biotinylated leucomyosuppressin (LMS), a myoinhibitory peptide hormone that affects gut contractions in insects, was prepared and fed to aphids. The avidin : biotin-LMS conjugate had insecticidal activity towards *A. pisum* when fed in diet at levels which neither of the components (avidin or biotin-LMS) caused significant mortality. It was hypothesised that binding to the gut, through the avidin moiety, was responsible for the observed oral toxicity of the avidin : biotin-LMS conjugate. The same principle was applied to lepidopteran larvae. A conjugate between avidin and biotinylated allatostatin was prepared and fed to *M. brassicae* larvae. The avidin : biotin-allatostatin was non-toxic, most likely due to cleavage between the biotin molecule and allatostatin as a result of the high levels of gut proteolysis in lepidoptera. Avidin conjugates of peptides that have little or no oral toxicity to insects, as a result of restricted access to sites of action, could have the potential to form a novel class of insecticidal compounds.

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Abbreviations

A	Amps (current)
AOX1	Alcohol oxidase
BCA	Bicinchoninic acid
bp	Base pairs (DNA)
BSA	Bovine serum albumin
ButaIT	Indian red scorpion toxin
°C	Degrees Celsius
Da	Dalton
DAP	1, 3-Diaminopropane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy (Base) triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetra-acetic acid
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
g	Grams
GAP	Glyceraldehyde-3-phosphate dehydrogenase
GNA	*Galanthus nivalis* agglutinin (Snowdrop Lectin)
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
IgG	Immunoglobulin G
kDa	Kilo Dalton
L	Litres
LAF	Laminar airflow
LB	Luria-Bertani
M	Molar
mg	Milligrams
mg/L	Milligrams per litre
ml	Millilitres
mm	Millimetres

mM	Millimolar
mRNA	Messenger ribonucleic acid
nm	Nanometres
nM	Nanomolar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNGase F	Peptide: N-Glycosidase F
ppm	Parts per million
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T_m	Melting temperature (of DNA)
TAE	Tris-Acetic acid-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
µg	Micrograms
µl	Microlitres
µm	Micrometres
UV	Ultraviolet
V	Volts (voltage)
(v/v)	Volume per volume
(w/v)	Weight per volume
(w/w)	Weight per total weight
YPG	Yeast extract-peptone-glycerol

DNA bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Amino Acid codes

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic Acid
C	Cysteine
E	Glutamic Acid
Q	Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

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Chapter 1

Introduction

Herbivorous Insect Pests – A Constraint on Agricultural Production

With the global population now reaching seven billion, there is an increased need to produce large amounts of food. However, a large constraint on meeting this requirement comes from crop spoilage by insects. The Global Crop Diversity Trust has recently estimated that as much as 30-40% of the world's crop produce is destroyed by agricultural pests and associated crop diseases. In the UK alone, more than 370 tonnes of insecticides are applied annually, with a cost now exceeding 25 million pounds (Garthwaite *et al.*, 2008).

There are over one million insect species worldwide, many of which are herbivores. However, the number of species that are serious agricultural pests is comparatively limited. The major pest species belong to the orders coleoptera, diptera, lepidoptera, hemiptera and orthoptera. These include both mono- or oligophagous herbivores (feeding on one or a limited range of closely related host plant species) and polyphagous herbivores (opportunistic feeders that will attack a wide range of plant species). Lepidopteran larvae are considered to be the most economically significant insect pests; therefore, almost half of all chemical insecticides are directed against species within that order (Brooks and Hines, 1999). Hemipteran insects also cause significant crop damage, not only because of the damage they cause whilst sap sucking, but also due to their role as vectors of plant diseases (Chrispeels and Sadava, 2003).

The current agricultural pest control strategy involves using large amounts of chemical insecticides. These include carbamates, neonicotinoids, organophosphates, synthetic pyrethroids, insect growth regulators and metabolic disruptors (Garthwaite *et al.*, 2008). However, there are significant problems and limitations associated with the widespread use of these conventional chemical insecticides. The most important of these problems, causing much public concern, is their broad-spectrum chemistry,

which gives a poor specificity to pest insect species and often causes toxicity to vertebrates. Over time, many insects that are beneficial to the environment have been harmed and ecosystems have been disrupted (Weisser and Siemann, 2004). This is why changes in European legislation (EU Directive 1991/414, EC 1107/2009) have led to the withdrawal of many chemical insecticides from commercial use.

With the loss of chemical insecticides, arable farmers have access to a reduced number of plant protection products. This, in turn, means that the extensive use of the fewer insecticides available rapidly increases the risk of insect resistance occurring. This is a crucial problem with similar chemical compounds such as pyrethroids for example, where resistance to one member of the pyrethroid family can mean a degree of resistance to others in that class (cross-resistance) (Beugnet and Chardonnet, 1995). Insect resistance to chemical pesticides has been common knowledge for many years. The occurrence has been steadily increasing and it is now reported that up to 1000 species of insects show resistance to one or more classes of insecticide (Miller, 2004). More importantly, for every class of chemical insecticide, there is at least one resistant insect (World Health Organisation, 1992). Beyond the extensive usage of these insecticides, the underlying reason for the increased development of insecticide resistance lies with the targets of the chemical insecticides. The majority of them aim to block or inhibit just one of five aspects of the central nervous system: voltage-gated sodium channels, γ-aminobutyric acid (GABA) receptors, nicotinic acetylcholine receptors (nAChRs), glutamate receptors and acetylcholinesterase (AChE) (Raymond-Delpech *et al.*, 2005). Insects have responded to this single target approach of crop protection by selecting for point-mutations in the sodium channel, within the ion channel of the GABA receptor, in the active site of the AChE, in the amplification of esterase genes, and for further mutations causing the up-regulation of detoxification enzymes (Feyereisen, 1995; Brogdon and McAllister, 1998; Hemingway and Ranson, 2000).

The non-target toxicity of chemical pesticides, the development of insect resistance to these pesticides, and the prospect of additional insect pests spreading into the UK as a result of environmental change, are the reasons why a more sustainable and environmentally sensitive approach to insect pest management is required.

Insecticidal Proteins and Protein Biopesticides: Alternative Methods of Insect Pest Control

Investigations into alternative approaches to the chemical control of insect pests has led to the development of protein-based technologies, some of which are described here.

Bacillus thuringiensis (*Bt*)

Bacillus thuringiensis (*Bt*) is a gram-positive, soil dwelling bacterium originally discovered by the Japanese biologist Ishiwata Shigetane in 1901. It was described as "a bacterium causing a disease affecting silkworms" (sudden-collapse). It was named *Bt* when Ernst Berliner encountered it in 1911 whilst studying its insecticidal activity towards *Anagasta kuehniella* (Mediterranean flour moth) (Lepidoptera: Pyralidae, Zeller 1879) (Berliner, 1911). Subsequent research showed that the insecticidal activity of *Bt* could be attributed to toxic proteins of the Cry, Cyt and Vip families (Angus, 1956). The Cry and Cyt proteins (δ-endotoxins) are produced by the bacterium upon sporulation and form components of a crystalline matrix associated with the spores, whilst the Vip proteins are expressed during the bacterial vegetative growth. The genes that encode the proteins are located on transmissible plasmids (Gonzalez *et al.*, 1982).

The three-domain Cry proteins have been extensively studied. They are named using a complex system based on similarities in their amino acid sequences (Crickmore *et al.*, 1998). Analysis of six Cry toxins by X-Ray crystallography allowed their tertiary domain structure to be characterised (Li *et al.*, 1991; Grochulski *et al.*, 1995; Li *et al.*, 1996; Galitsky *et al.*, 2001; Morse *et al.*, 2001; Boonserm *et al.*, 2005). Generally, the N-terminal domain I consists of seven anti-parallel α-helices, where helix five is hydrophobic and is therefore contained in the centre of the other six. Domain II contains three anti-parallel β-sheets arranged in a β-prism fold. The C-terminal domain III is a β-sandwich created from two twisted, anti-parallel β-sheets. Each domain plays a specific role in the toxins activity (see next page).

The insecticidal activity of the three-domain Cry toxins occurs in a three-step process. First, the protoxins are activated by proteolysis. Following ingestion, the protoxins are solubilised and undergo proteolytic cleavage that removes fragments from both the C- and N-terminals (Milne and Kaplan, 1993). Domain III is believed to be involved in insect specificity (Aronson *et al.*, 1995; Lee *et al.*, 1995). Secondly, the activated toxin binds to receptors in the gut epithelium and a conformational change promotes its insertion into the cell membrane (Hoffman *et al.*, 1988; Bravo *et al.*, 1992; Knowles and Dow, 1993). Domain II is responsible for the specific receptor binding, as discovered by mutagenesis (Schnepf *et al.*, 1998). Finally, the toxin is oligomerised. This then forms a pore causing osmotic cell lysis (Sacchi *et al.*, 1986; Wolfersberger, 1989; Lorence *et al.*, 1995; Bravo *et al.*, 2004). Domain I shows similarity to other bacterial pore-forming toxins (Gazit *et al.*, 1998) and is believed to form the oligomer that constitutes the open channel in this final step (Bravo *et al.*, 2007).

The δ-endotoxins (Cry and Cyt proteins) are considered safe for use as insecticides because the protoxins are highly insoluble in the gut conditions of humans and other vertebrates and there are no receptors for the toxins in mammalian gut cells. Thus, they have been used in biopesticides since 1920. However, *Bt* biopesticides account for less than 1% of the annual pesticide usage because, to prevent them from being washed off by rainfalls, they must be applied with extreme care. They are also susceptible to degradation in the presence of UV light (Pusztai *et al.*, 1991).

As an alternative to *Bt* sprays, *Bt* transgenic tobacco, maize, corn, potato, tomato, cotton and rice crops have been produced (Delannay *et al.*, 1989; Perlak *et al.*, 1990; Tian *et al.*, 1991; Koziel *et al.*, 1993; Perlak *et al.*, 1993; Datta *et al.*, 1998). Following their approval in the US by the Environmental Protection Agency in 1995, these commercialised plants have been some of the most successful genetically modified (GM) crop products: with the *Bt* toxins conferring protection from insect pests, insecticide usage on these crops has been reduced by 60% (Shelton *et al.*, 2000; Huang *et al.*, 2002; Ismael *et al.*, 2002).

Bt transgenic plants are not protected from sap-sucking hemipteran pests however. These possess the *Bt* receptors required for insecticidal activity, but it is believed that their low levels of digestive proteolysis only gives a small amount of active toxin build-up (Cristofoletti *et al.*, 2006). There are also some other problems and limitations with *Bt* transgenic crops, namely, the potential impact of *Bt* toxins on non-target species containing *Bt* receptors within their gut, an increase in the *Bt* toxin levels in the soil which could affect soil organisms before the toxins degrade, the exchange of genetic material between plants, and the development of insect resistance (Kumar, 2002). Since their first use in *Bt* sprays, more than seven species of insect have developed resistance to *Bt* toxins (Marrone and Macintosh, 1993; Tabashnik *et al.*, 2009). The first occurrence of insect resistance to *Bt* transgenic crops has recently been confirmed in India (Bagla, 2010).

Methods to delay the development of insect resistance have been researched and employed since the crops were introduced. These approaches include:

(i) Gene pyramiding. Two or more Cry toxins are expressed in transgenic plants, so that insects would need to develop resistance to more than one toxin to survive. The odds of insects developing resistance to two toxins simultaneously are considerably lower than the odds of insects developing resistance to a single toxin.

(ii) Targeted expression of the *Bt* toxins. Only allowing the plant to express the toxins when their presence is required reduces the time the insects are exposed to the toxin.

(iii) A refuge strategy. This involves growing a small area of non-transgenic crops to maintain a refuge where insects without resistance mutations can reside and reproduce, therefore maintaining non-resistant genes within the insect gene pool and discouraging selection pressure in favour of resistant individuals.

(iv) High dosage delivery of multiple *Bt* toxins. Using high dosages of δ-endotoxins in transgenic crops will ensure that virtually all pests are killed, reducing the chances of different Cry toxin resistant insects breeding with each other.

Despite the success of *Bt* transgenic crops, their limitations in the range of pests targeted and the potential for the development of resistance has meant that other biopesticides still need to be developed for sustainable protection of crops.

More recently, a *Bt* toxin fusion protein with the galactose-binding domain of the ricin B-chain has been expressed successfully (Mehlo *et al.*, 2005), highlighting the fact that combinations of different toxins can act synergistically and may be a further method to combat insect resistance developing.

Other Bacterial Toxins

1. Cholesterol Oxidase

Bacterial cholesterol oxidase is an enzyme that catalyses the oxidation of cholesterol. In terms of insect control, it has an insecticidal activity comparable to that of *Bt* toxins. Corbin *et al.* (2001) established that when the enzyme was expressed and localized in chloroplasts of tobacco, the leaf tissue was toxic to *Anthonomus grandis* (boll weevil) (Coleoptera: Curculionidae, Boheman 1843) larvae without any adverse effects on the phenotype of the plant.

2. *Photorhabdus luminescens* Insecticidal Proteins

Photorhabdus luminescens is a symbiotic bacterium found in nematodes of *Heterorhabditis* species. When the nematodes enter a host insect, the bacterial cells are released from the nematode and enter the insect circulatory system. The bacteria secrete toxins that cause host cell death, leading to a lethal septicaemia. *Photorhabdus luminescens* bacteria contain multiple insecticidal toxins. Liu *et al.* (2003) isolated and expressed one of the toxins (toxin A) in leaves of *Arabidopsis thaliana*, which was found to confer almost complete protection against *Manduca sexta* (tobacco hornworm) (Lepidoptera: Sphingidae, Linnaeus 1763) and *Diabrotica virgifera* (corn rootworm) (Coleoptera: Chrysomelidae, LeConte 1868).

Plant Defensive Proteins

1. Protease Inhibitors (PIs)

Protease inhibitors (PIs) are small proteins, ubiquitous in nature, that vary in size from around 5kDa to 20kDa (Hung *et al.*, 2003). Their function is to reversibly or irreversibly block or alter the active site of their target enzymes. They are believed

to be involved in the plant defence system; being produced in seeds and specific plant tissues upon wounding by insect herbivores (Koiwa *et al.*, 1997). Once consumed by a herbivore, the plant PIs affect the activity of digestive enzymes within the insect gut, therefore altering the quantity of proteins that can be digested. Plant PIs include a number of different families of proteins, targeting all of the major types of digestive protease activity (serine, cysteine, aspartic and metallo-proteases).

Mickel and Standish (1947) originally described the role of PIs in plant defences after noticing how the development of various insect larvae was retarded following feeding on soybean products. Subsequently, soybean trypsin inhibitors were shown to cause retardation of development and toxicity towards *Tribolium confusum* (flour beetle) (Coleoptera: Tenebrionidae, Jacquelin du Val 1863) larvae (Lipke *et al.*, 1954). Since then, over 500 PIs have been isolated, mostly from the *Leguminosae*, *Poaceae* and *Solanaceae* families (Brzin and Kidric, 1995) and are listed in the PLANT-PI database (De Leo *et al.*, 2002).

The first transgenic plant expressing a foreign PI, the cowpea trypsin inhibitor (CpTI), was produced in 1987 (Hilder *et al.*, 1987). The transgenic tobacco showed resistance to *Heliothis virescens* (tobacco budworm) (Lepidoptera: Noctuidae, Fabricius 1777) larvae, resulting in increased mortality and decreased feeding. Transgenic plants are relatively easy to produce, as only a single PI gene needs to be activated. A whole range of PI transgenic plants have therefore been produced, including transgenic rice, tomato, potato, cotton and wheat (Orozco-Cardenas *et al.*, 1993; Gatehouse *et al.*, 1996; Li *et al.*, 1998; Altpeter *et al.*, 1999; Lee *et al.*, 1999). Besides protection from herbivorous insects, PI transgenic plants have also been shown to be effective against nematodes, viruses and fungal infections (Lorito *et al.*, 1994; Vain *et al.*, 1998; Gutierrez-Campos *et al.*, 1999; Urwin *et al.*, 2000).

Although the successful production of PI transgenic plants is well documented, there have also been reports of transformed plants expressing PIs but showing little or no insect resistance (Johnson *et al.*, 1989; McManus *et al.*, 1994; Wu *et al.*, 1997; Altpeter *et al.*, 1999; Cloutier *et al.*, 2000). Some insects overcome the effects of the expressed PIs by inducing alternative proteolytic enzymes (Bolter

and Jongsma, 1995; Jongsma *et al.*, 1995), or by producing proteases that can themselves digest PIs (Girard *et al.*, 1998). Despite these resistance issues and other concerns regarding the toxicity of PIs towards non-target organisms (Malone *et al.*, 1995), a cowpea trypsin inhibitor has recently been successfully employed commercially in cotton in China (Cui, 2003).

2. Lectins

Lectins are a large and diverse group of proteins ubiquitous in nature that have been shown to bind carbohydrates and thereby agglutinate cells (agglutinins) (Pusztai, 1991). They contain a non-catalytic domain that can bind specifically but reversibly to mono- and oligosaccharides (Peumans and Van Damme, 1995). Some common examples are listed in Table 1.1. Many more lectins exist however. The true role of lectins in plants is still unknown, however, roles in growth regulation, legume nodulation and plant defence have been proposed (Roberts and Goldstein, 1983; Gatehouse *et al.*, 1995; Hirsch, 1999).

Lectin	Details
Snowdrop Lectin (GNA)	Mannose binding lectin purified from *Galanthus nivalis*
Concanavalin A (Con A)	Mannose binding lectin purified from *Canavalia ensiformis*
Garlic Lectin (ASA)	Mannose binding lectin purified from *Allium sativum*
Phytohaemagglutinin (PHA)	Mannose binding lectin purified from Legumes
Ricin (RCA)	Consists of a Galactose binding lectin purified from *Ricinus communis*
Peanut Agglutinin (PNA)	Galactose binding lectin purified from *Arachis hypogaea*
Wheat Germ Agglutinin (WGA)	N-acetylglucosamine binding lectin purified from *Triticum vulgaris*

Table 1.1.
Examples of plant lectins.

Many lectins are insecticidal, with effects recorded in species of coleoptera, homoptera and lepidoptera (Czapla and Lang, 1990; Murdock *et al.*, 1990; Powell *et al.*, 1995; Rhabe *et al.*, 1995; Gatehouse *et al.*, 1999). However, some lectins such as

phytohaemagglutinin (PHA) have also been shown to be toxic to mammals (Pusztai *et al.*, 1995).

Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) is an extensively studied lectin. It is a D-mannose-specific binding lectin isolated from the bulbs of snowdrops (Van Damme *et al.*, 1987). Snowdrop lectin has a tetrameric structure with each identical subunit being 12.5kDa in size. The subunits can each bind three mannose sugar residues (Van Damme *et al.*, 1998). Powell *et al.* (1993) first established the insecticidal activity of GNA in *Nilaparvata lugens* (rice brown plant hopper) (Homoptera: Delphacidae, Stål 1854). Since then, GNA transgenic tobacco, rice, wheat and potatoes have been produced and tested for herbivore resistance (Zhou *et al.*, 1998; Rao *et al.*, 1998; Bell *et al.*, 2001; Liang *et al.*, 2004). The transgenic plants were less susceptible to insect attack.

3. *Phaseolus vulgaris* (bean) α-amylase Inhibitor

The α-amylase inhibitors isolated from legume seeds have been shown to confer resistance to coleopteran seed weevils. *Phaseolus vulgaris* (bean) α-amylase inhibitor was expressed in *Pisum sativum* (garden pea) seeds and their resistance to *Bruchus pisorum* (pea weevil) (Coleoptera: Bruchidae, Linnaeus 1758) and other bruchid beetle larvae was demonstrated (Shade *et al.*, 1994; Morton *et al.*, 2000). The use of α-amylase inhibitors for their insecticidal properties is restricted to insects, mostly coleopterans, which have a neutral or acidic gut pH to maintain the inhibitor in its active state. As with other plant defence proteins, widespread commercialisation has not taken place due to safety concerns within higher animals (Prescott *et al.*, 2005).

Insecticidal Toxins from Arthropods

Many arthropods feed on insects and use insecticidal toxins as a means of immobilising or killing their prey. These insecticidal toxins are commonly constituents of venom produced by the arthropod. Scientific research has shown that venoms can be reservoirs of insecticidal toxins, often containing complex mixtures of polypeptides that can induce paralysis, disrupt the development of the prey and interfere with its immune response, reproductive system or metabolic regulation

(Altstein, 2004: Gade, 2004; Tedford *et al.*, 2004; Gade and Hoffmann, 2005). Because of these effects, venom-derived toxins have the potential to be used as insecticides. Whilst many arthropods also sting and bite humans and higher organisms, the small amounts of venom injected in comparison to their large size means that most are non-lethal. Insect-specific toxins can be selected, as can toxins that show specificity to a range of target species.

Below are two examples of toxins chosen for their documented specific activity against lepidopteran larvae (a major worldwide pest). Many more arthropod toxins exist.

1. A Toxin from *Mesobuthus tamulus* (Indian Red Scorpion)

Wudayagiri *et al.* (2001) isolated an insecticidal toxin from the venom of *Mesobuthus tamulus* (Indian red scorpion) (Scorpiones: Buthidae, Fabricius 1798) and named it ButaIT. Mature ButaIT is a single-chain 37 amino acid polypeptide consisting of an α-helix and three β-strands with four disulphide bridges. It is classified as a short insect toxin (30-40 amino acids) due to its high sequence homology with other toxins that affect voltage dependent potassium channels and large conductance calcium activated potassium channels (Carbone *et al.*, 1982). It was originally entered into databases (Genbank accession number AF481881, Swiss-prot number P81761) as a novel lepidopteran-selective toxin, showing high specificity to *Heliothis virescens* (tobacco budworm) (Lepidoptera: Noctuidae, Fabricius 1777) larvae, inducing a progressive, irreversible flaccid paralysis leading to eventual larval mortality (at 1μg/100mg larvae). No toxic effects were observed in *Sarcophaga falculata* (blowfly) (Diptera: Calliphoridae, Pandelle 1896) larvae or mice (Wudayagiri *et al.*, 2001).

2. A Toxin from *Hadronyche versuta* (Australian Funnel Web Spider)

Fletcher *et al.* (1997) isolated the ω-atracotoxin-Hv1a toxin from the venom of *Hadronyche versuta* (Australian funnel web spider) (Araneae: Hexathelidae, Rainbow 1914). The ω-atracotoxin-Hv1a toxin, a member of the ω-ACTX-1 family of insecticidal toxins, is a 37 amino acid polypeptide consisting of a β-turn core region containing three disulphide bridges and a β-hairpin that protrudes from this

core. It was shown to block neural transmission in cockroaches and was characterised as an insect-specific voltage-gated calcium channel agonist (Fletcher *et al.*, 1997).

Insecticidal Neuropeptides from Arthropods

Many vital processes in arthropods are specifically regulated by peptides and hormones produced internally. These can be stimulatory or inhibitory. Introducing or adjusting the amounts of these peptides and hormones therefore has a subsequent effect on the process, which can ultimately be fatal. Insect-specific neuropeptides can be selected, as can neuropeptides that show specificity to a range of target species.

Below are two examples of neuropeptides chosen for their documented specific activity against insect gut muscular contractions and motility (a vital process for crop insect pests). Many more arthropod neuropeptides exist.

1. A Neuropeptide from *Leucophaea maderae* (Cockroach)

Holman *et al.* (1986) isolated an inhibitory neuropeptide from *Leucophaea maderae* (cockroach) (Orthoptera: Blaberidae, Fabricius 1781). Leucomyosuppressin (LMS) is a simple 10 amino acid FMRFamide-related peptide with the sequence pEDVDHVFLRFamide. The FMRFamide peptides occur quite widely in nature and are known to be involved in regulation of heart rate, blood pressure, gut motility, feeding behaviour and reproduction (Kingan *et al.*, 1990; Kingan *et al.*, 1996; Predel *et al.*, 2001; Aguilar *et al.*, 2004; Audsley and Weaver, 2006). The LMS peptide acts on gut muscular contractions (gut peristalsis). When LMS was injected into cockroaches, an accumulation of food within the foregut and a decrease in food consumption was observed as a result of inhibition of gut peristalsis (Aguilar *et al.*, 2004). Similar effects were seen in *Lacanobia oleracea* (tomato moth) (Lepidoptera: Noctuidae, Linnaeus 1758) and *Spodoptera littoralis* (cotton leafworm) (Lepidoptera: Noctuidae, Fabricius 1775) (Matthews *et al.*, 2008).

2. A Neuropeptide from *Manduca sexta* (Tobacco Hornworm)

Kramer *et al.* (1991) first identified a C-type neuropeptide from *Manduca sexta* (tobacco hornworm) (Lepidoptera: Sphingidae, Linnaeus 1763). This new type of allatostatin (Manse-AS) was structurally distinct from the A- and B-types isolated

in cockroaches and crickets (Woodhead *et al.*, 1989; Pratt *et al.*, 1989; Lorenz *et al.*, 1995). The Manse-AS neuropeptide is a relatively simple peptide with the sequence QVRFRQCYFNPISCF, which is known to inhibit gut contractions (Kramer *et al.*, 1991).

Recently, a teratocyte secretory protein gene from an endoparsite has been expressed in transgenic plants (Maiti *et al.*, 2003), however, the main restriction around using insecticidal toxins and neuropeptides from arthropods as a method of insect pest control is the mode of delivery and the target location of the toxin or neuropeptide. Many species of insect have differing gut morphologies and pH conditions where orally delivered toxins and neuropeptides are susceptible to proteolytic degradation (Lehane and Billingsley, 1996).

Avidin as an Insecticidal Protein

Avidin is a protein found in the egg white of birds, reptiles and amphibians that was originally discovered by Esmond Emerson Snell (Eakin *et al.*, 1940). It is a basic glycoprotein, with an isoelectric point (pI) of 10.0 (Woolley and Longsworth, 1942). It is thought to function as an antibacterial host-defence protein by virtue of its ability to bind biotin, an essential vitamin for most organisms (vitamin B_7 or vitamin H). In nature, avidin exists as a homotetramer, comprised of four 128 amino acid subunits (16kDa), each singly glycosylated and able to bind one biotin molecule with extreme specificity and affinity (DeLange and Huang, 1971; Green, 1990). The avidin-biotin dissociation constant is 10^{-15} (Savage *et al.*, 1992).

The insecticidal activity of avidin was first determined 50 years ago, when dietary avidin was found to have detrimental effects on the growth of *Musca vicina* (housefly) (Diptera: Calliphoridae, Macquart 1851) larvae (Levinson and Bergmann, 1959). Avidin, and the related bacterial protein streptavidin, have since been shown to be toxic to a wide range of insects including representatives of lepidoptera (Morgan *et al.*, 1993; Du and Nickerson, 1995; Markwick *et al.*, 2001; Burgess *et al.*, 2002; Cheng Zhu *et al.*, 2005), diptera (Levinson and Bergmann, 1959; Tsiropoulos, 1985; Bruins *et al.*, 1991), coleoptera (Levinson *et al.*, 1967; Allsopp and McGhie, 1996; Kramer *et al.*, 2000; Yoza *et al.*, 2005; Cooper *et al.*, 2006; Murdock and Shade, 2008) and orthoptera (Christeller *et al.*, 2000).

Avidin is a normal component of human diet. It therefore has the potential to be adopted as an insect control agent because the dietary levels of the protein that are required to provide effective insecticidal activity against a wide range of pests are much lower than the levels present in normal human diet. Expression of avidin in transgenic plants has been put forward as a strategy to confer protection against insect pests, with engineering of maize, corn, tobacco, potato, apple and rice being reported (Kramer *et al.*, 2000; Burgess *et al.*, 2002; Murray *et al.*, 2002; Markwick *et al.*, 2003; Yoza *et al.*, 2005). However, the initial report of expression of avidin in maize with the intention of producing protein that could be extracted for use as a biochemical reagent (Hood *et al.*, 1997) remains the only example of large-scale expression *in planta*.

The insecticidal effects of avidin are mediated through its biotin-binding activity. In many cases, these effects were shown to be eliminated by feeding diets supplemented with biotin, supporting the hypothesis that avidin acts through the sequestration of biotin from ingested food, thereby preventing absorption from the gut and causing a biotin deficiency in the insect (Kramer *et al.*, 2000). Biotin, as a cofactor of major carboxylases involved in key processes such as gluconeogenesis, lipogenesis, and fatty acid and amino acid catabolism, is essential for insect growth (Wood and Barden, 1977; Knowles, 1989). Insects fed on avidin-containing diets show retarded growth, eventually leading to mortality. Variability in susceptibility to the toxic effects of avidin is thought to depend on variations in levels of maternally-derived biotin (Markwick *et al.*, 2001). Perhaps a reason why transgenic avidin crops have not been commercialised is that insects could potentially increase the levels of maternally-derived biotin to overcome the effects of avidin expressed in crops.

Protein Transport Across the Insect Gut

In order to be able to exploit many insecticidal proteins as biopesticides, a technical problem must be solved. Whetstone and Hammock (2007) note, "considering the total number of species that produce insect-specific toxins and the variety of toxins within each venom type, the potential for the development and application of novel biopesticides from these sources appears virtually limitless –

limitless, however, only if provided with suitable delivery systems." Therefore, the problem with protein-based technologies is not in finding insecticidal proteins, but finding a suitable delivery method for these proteins.

Many insecticidal proteins that are active when directly injected into the insect are unlikely to pass through an insect cuticle by themselves. They are usually non-toxic even when administered orally, as they will not cross the gut wall to access their sites of action, which include for instance, ion channels in nervous tissue. For this reason, besides the insecticidal properties of lectins described earlier, their carbohydrate-binding specificity is of interest to scientists today. Many gut membrane proteins such as brush-border enzymes, receptors and transport proteins are glycosylated, producing potential lectin binding sites. It is the binding at these glycosylated sites that is thought to be at the centre of the ability of certain lectins to translocate across the gut epithelium into the haemolymph (Boyd and Reguera, 1949; Liener *et al.*, 1986). The presence of intact snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) and jackbean lectin (*Canavalia ensiformis* concanavalin A; Con A) within the haemolymph of *Lacanobia oleracea* (tomato moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae following oral delivery has been demonstrated (Fitches *et al.*, 2001). Similar behaviour has also been observed for other lectins (Fitches *et al.*, 2008).

Fusion Proteins

Creating fusion proteins by combining two or more proteins has recently become a widespread technique among researchers. The components of fusion proteins act together to produce effects that are not observed when they act independently. Fusion proteins could therefore be useful in addressing issues relating to protein delivery.

Synthetic Fusion Proteins as Insecticides

The fusion protein approach has been used to develop a novel method of delivering insect-specific, biologically active peptides to the haemolymph of insect pests following oral ingestion. The technology utilizes recombinant DNA techniques

to fuse a coding sequence for an insect-derived, toxic peptide to that of a plant lectin. The resulting fusion protein is then produced by recombinant expression in the methylotrophic yeast *Pichia pastoris*. When fed to target insects via artificial diet, the plant lectin binds and subsequently translocates across the insect gut epithelium, acting as a 'carrier,' delivering the peptide to the haemolymph of the insect where it can exert a toxic effect (Figure 1.1).

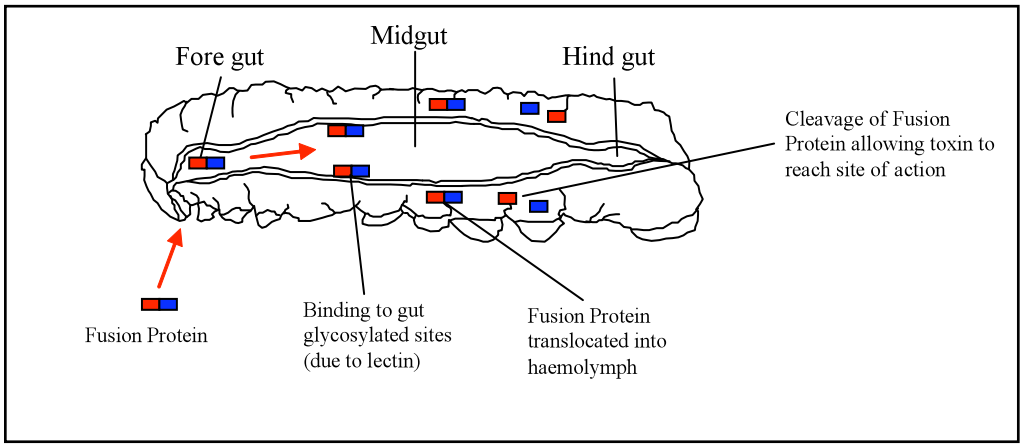


Figure 1.1.
Schematic representation of a lepidopteran larva.
The fate of an orally delivered fusion protein.

To date, this synthetic fusion protein technology has been exploited to produce novel recombinant C- and N-terminal fusion proteins with snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) using *Manduca sexta* (tobacco hornworm) (Lepidoptera: Sphingidae, Linnaeus 1763) allatostatin, *Segestria florentina* (tube-web spider) (Araneae: Segestriidae, Rossi 1790) toxin and *Mesobuthus tamulus* (Indian red scorpion) (Scorpiones: Buthidae, Fabricius 1798) toxin. All three of the fusion proteins have demonstrated promising results, with oral toxicity to lepidopteran insect larvae demonstrated (Fitches *et al.*, 2002; Fitches *et al.*, 2004; Pham-Trung *et al.*, 2006).

Employing a mannose-binding lectin as the 'carrier' ensures a fusion protein is instantly specific for organisms containing glycoproteins or lipopolysaccharides with high-mannose carbohydrate side chains within their guts. These are relatively

uncommon in higher animals. The use of an insect-specific or insect order-specific toxic peptide also increases the specificity to a particular insect or to an insect order. Furthermore, it is thought unlikely that fusion proteins would be harmful to other non-target organisms, such as beneficial insects and mammals, as cleavage of fusion proteins may occur once they are transferred into the haemolymph of the target insect. This cleavage would allow the insecticidal protein to reach its site of action, but as it would no longer be associated with the lectin, it would mean that it would not be carried to the haemolymph of any other predator, should the target insect be consumed.

The main limitation of using GNA as the fusion protein 'carrier' is the public perception that it is toxic to higher animals following the Pusztai affair (Ewen and Pusztai, 1999; Rhodes, 1999).

Synthetic fusion protein technology offers great potential for the development of further insect-targeted, environmentally benign insecticides from peptides that are otherwise ineffective when administered alone due to either degradation by digestive enzymes within the insect gut or failure to permeate the gut membrane. With continued scientific and industrial research, the technology may provide, in part, an answer to some of the problems associated with current insecticides and with the threat of insect pest problems arising as a result of global environmental change.

Conjugates as Insecticides

Besides fusing peptides together using recombinant DNA techniques, covalent linkage or non-covalent interactions between proteins and peptides can be used to produce conjugates. Conjugates are frequently used for purification in research and in medical treatments, but there are no published examples of using conjugates as insecticides.

Advantages of Avidin as a Basis for Constructing Fusion Proteins and Conjugates

Like GNA, avidin has recently been shown to be translocated into the haemolymph of lepidopteran larvae following oral delivery (Hinchliffe, 2007). This property makes avidin a suitable candidate for use as a 'carrier' protein in synthetic fusion protein technology to produce recombinant insecticidal fusion proteins: the

avidin could be used in the same way as the GNA within the GNA fusions described earlier. Using avidin as a 'carrier' protein may resolve the public perception limitation with GNA fusion proteins. Avidin also has the advantage of having a biotin-binding site and it has previously been shown that biotin remains bound to avidin upon transfer to the haemolymph of lepidopteran larvae (Hinchliffe, 2007). This means that besides being a potential 'carrier' in synthetic fusion protein technology, avidin could also be utilised to attach biotinylated peptides to the biotin-binding site and produce an insecticidal conjugate. Using avidin as a 'carrier' protein or as part of a conjugate would be advantageous over using it solely as a biopesticide to sequester dietary biotin, because it would eliminate the chance of resistance developing due to an increase in the levels of maternally-derived biotin in insects.

Expression Systems for Recombinant Proteins Based on *Pichia pastoris*

Since *Pichia Pastoris*, a methylotrophic yeast, was established as an organism for heterologous protein expression (Ogata *et al.*, 1969), numerous vectors, strains and manipulation protocols have been developed (Cereghino and Cregg, 2000). It is now frequently used for the following reasons:

(i) In spite of being a single-celled eukaryotic micro-organism, it is capable of many post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulphide bond formation and glycosylation.

(ii) It is fast and easy to manipulate.

(iii) It can withstand the high cell densities involved in culturing therefore giving higher protein yields.

Many proteins that express as insoluble inclusion bodies in *Escherichia coli* have been successfully expressed in yeast (Cereghino and Cregg, 2000).

A large range of expression vectors are available for *P. pastoris*, with the two main types being those containing inducible promoters, such as the alcohol oxidase 1 gene (*AOX1*) promoter, and those containing constitutively expressed promoters, such as that from the glyceraldeyde-3-phosphate dehydrogenase gene (*GAP*) (Waterham *et al.*, 1997). In addition, vectors can contain the *Saccharomyces*

cerevisiae alpha factor secretion signal prepropeptide to direct proteins to the secretory pathway (Duntze *et al.*, 1970). This therefore allows the user to easily select between inducible or constitutive expression of heterologous protein and intracellular expression or secretion into the extracellular media. Extracellular secretion aids the protein purification process because *P. pastoris* itself secretes very low levels of endogenous proteins and the culture medium does not contain any added proteins either. Secreted heterologous protein therefore comprises the majority of the protein in the external medium and is already separated from cellular proteins. However, wild-type *P. pastoris* strains, for example X33, have a tendency to release aspartic proteases, and recombinant proteins can be undesirably cleaved by endogenous protease activity. This can be kept to a minimum by utilising protease deficient *P. pastoris* strains such as SMD1168, but secreted protein cleavage has yet to be eliminated completely (Jahic *et al.*, 2006). The use of *P. pastoris* as an expression host has been found to be essential to produce functionally active insecticidal fusion proteins (Fitches *et al.*, 2004)

Project Aims and Objectives

The aim of this project is to investigate the use of recombinant avidin for the protection of crops against insect pests. Specifically, the objectives are:

1. To analyse recombinant avidin produced using *Pichia pastoris*. In particular, to study its *in vitro* activity in comparison to commercially available avidin and to test its activity *in vivo* towards hemipteran pests.

2. To investigate the use of avidin as a 'carrier' for toxic peptides in synthetic fusion protein technology by producing recombinant avidin fusion proteins and analysing their insecticidal activity.

3. To establish if recombinant avidin can be utilised to produce insecticidal conjugates with toxic peptides.

Chapter 2

Materials and Methods

Biological and Chemical Reagents

All chemicals and reagents utilised were supplied by VWR (Radnor, USA) (BDH grades), Sigma (St. Louis, USA), or Merck (Whitehouse Station, USA) unless otherwise stated. These were of laboratory grade or higher.

For bacterial work, One Shot TOP10 Electrocomp *Escherichia coli* cells of the genotype F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G were used (supplied by Invitrogen, Carlsbad, USA). Aliquots of 50μl were stored at -80°C.

For yeast work, Easycomp protease-deficient SMD1168H of the genotype *pep*4 and X33 wild-type strains of *Pichia pastoris* were used (supplied by Invitrogen). Aliquots of 50μl were stored at -80°C.

Frequently Used Solutions

Bacterial Culture Media

- Low Salt LB Media and Agar

  1% (w/v) trypticase peptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride.

  Low salt LB agar for petri dishes is identical to the above with the addition of 1.5% (w/v) agar (supplied by Difco, Franklin Lakes, USA).

Yeast Culture Media

- YPG Media and Agar

  2% (w/v) trypticase peptone, 1% (w/v) yeast extract, 4% (v/v) glycerol.

  YPG agar for petri dishes is identical to the above with the addition of 2% (w/v) agar (supplied by Difco).

Fermentation Media

26.7ml/L 85% phosphoric acid

0.93g/L calcium sulphate

18.2g/L potassium sulphate

14.9g/L magnesium sulphate 7-hydrate

4.13g/L potassium hydroxide

40g/L glycerol

- PTM1 Trace Salts

6g/L Cupric sulphate 5-hydrate

0.08g/L Sodium iodide

3g/L Manganese sulphate hydrate

0.2g/L Sodium molybdate 2-hydrate

0.02g/L Boric acid

0.5g/L Cobalt chloride

20g/L Zinc chloride

65g/L Iron(II) sulphate 7-hydrate

0.2g/L Biotin

5ml/L Sulphuric acid

Agarose Gel Electrophoresis

- 50x TAE

2M tris-HCl, 2M glacial acetic acid, 50mM ethylenediaminetetra-acetic acid pH 8.0.

- Orange G Loading Dye

10mM tris-HCl pH 7.6, 0.15% (w/v) Orange G, 60% (v/v) glycerol, 60mM ethylenediaminetetra-acetic acid.

- Lambda DNA/Eco47I Size Marker (supplied by Fermentas, Waltham, USA)

Protein Gel Electrophoresis

- Resolving Gel

15% (v/v) acrylamide, 0.2M tris-HCl pH 8.8, 0.1% sodium dodecyl sulphate, 0.1% (w/v) ammonium persulphate, 0.5% (v/v) N, N, N′, N′-tetramethylethylene-diamine.

The percentage of acrylamide (supplied by National Diagnostics, Atlanta, USA) is varied as required (normally 12.5%, 15%, 17.5% or 20%).

- Stacking Gel

4% (v/v) acrylamide, 0.13M tris-HCl pH 6.8, 0.1% (w/v) sodium dodecyl sulphate, 0.1% (w/v) ammonium persulphate, 0.75% (v/v) N, N, N′, N′-tetramethylethylene-diamine.

- 10x Reservoir Buffer

0.25M tris-HCl, 1.92M glycine, 1% (w/v) sodium dodecyl sulphate.

- 5x SDS-PAGE Loading Dye

312.5mM tris-HCl pH 6.8, 50% (v/v) glycerol, 10% (w/v) sodium dodecyl sulphate, 0.01% (w/v) Bromophenol Blue, 25% (v/v) 2-mercaptoethanol.

- Coomassie Stain

0.04% (w/v) Coomassie Brilliant Blue, 40% (v/v) methanol, 7% (v/v) glacial acetic acid.

- Destain

40% (v/v) methanol, 7% (v/v) glacial acetic acid.

Silver Staining Reagents:

- Fixative Solution

40% (v/v) ethanol, 10% (v/v) glacial acetic acid.

- Wash Solution

30% (v/v) ethanol.

- Thiosulphate (Sensitizer) Solution

0.02% (w/v) sodium thiosulphate.

- Silver Nitrate Solution

0.2% (w/v) silver nitrate, 0.02% (v/v) formaldehyde (37% solution).

- Developing Solution

3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde (37% solution), 0.0005% (w/v) sodium thiosulphate.

- Stop Solution

0.5% (w/v) glycine.

- SDS 7 Protein Size Marker (supplied by Sigma)

66kDa Bovine albumin

45kDa Egg albumin

36kDa Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)

29kDa Bovine carbonic anhydrase

24kDa Bovine pancreas trypsinogen

20.1kDa Soybean trypsin inhibitor

14.2kDa Bovine milk α-Lactalbumin

Western Blotting

- Bejerrum and Schafer-Nielsen Semi-Dry Transfer Buffer

48mM tris-HCl, 39mM glycine, 20% (v/v) methanol.

- Ponceau S Stain

0.5% (w/v) Ponceau S, 1% (v/v) glacial acetic acid.

- 10x Phosphate Buffered Saline

0.2M phosphate, 1.5M sodium chloride.

- Antisera Solution (Block)

  5% (w/v) non-fat milk powder, 1x phosphate buffered saline, 0.05% (v/v) Tween-20.

- Rinse Solution (PBS-Tween)

  1x phosphate buffered saline, 0.05% (v/v) Tween-20.

- ECL Solution

  1M tris-HCl pH 8.5, 0.2mM coumaric acid, 1.25mM luminol + 3% (v/v) hydrogen peroxide.

Column Chromatography

- SP Sepharose Column Loading Buffer

  50mM sodium acetate, pH 4.0.

- SP Sepharose Column Elution Buffer

  50mM sodium acetate, 1M sodium chloride, pH 4.0.

- Nickel Column Binding Buffer

  50mM sodium acetate, 0.5M sodium chloride, pH 4.0.

- Nickel Column Elution Buffer

  50mM sodium acetate, 300mM imidazole, 0.5M sodium chloride, pH 7.4.

- Phenyl Sepharose Column Loading Solution

  2M sodium chloride.

- Phenyl Sepharose Column Elution Solution

  Purified water.

Insect Cultures

Insect cultures originally obtained from the Food and Environment Research Agency (FERA), Sand Hutton, York, UK were subsequently maintained at Durham University.

Mamestra brassicae (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) were reared continuously on artificial diet (Bown *et al.*, 1997, see below) under controlled environmental conditions of 23°C, 40% relative humidity and a 16 hour light: 8 hour dark regime.

Sitobion avenae (cereal aphid) (Hemiptera: Aphididae, Fabricius 1775) were kept on two week old oat plants (*Avena sativa*) under controlled environmental conditions of 18°C, 30% relative humidity and a 16 hour light: 8 hour dark regime.

Acyrthosiphon pisum (pea aphid) (Hemiptera: Aphididae, Harris 1776) were maintained on established broad bean plants (*Vicia fabae*) under controlled environmental conditions of 18°C, 30% relative humidity and a 16 hour light: 8 hour dark regime.

Insect Diet

- *Mamestra brassicae* Artificial Diet

74g/L Haricot bean flour
59g/L Wheat germ
30g/L Soyabean flour
20g/L Casein
33.4g/L Brewers yeast
3.6g/L Ascorbic acid
1.8g/L Sorbic acid
3g/L Methyl 4-hydroxybenzoate
8.4g/L Vanderzant vitamin mixture
6.7g/L Wesson salt mixture
0.12g/L Ampicillin sodium salt
3.7ml/L Formaldehyde (37% solution)
1.4ml/L Linseed oil
13.4g/L Agar
(Produced as described in Bown *et al.*, 1997).

- Hemipteran Artificial Diet

Amino Acid Stock (per 50ml)

50.8mg Alanine

213.9mg Asparagine

189.7mg Aspartate / Aspartic acid

42.5mg Cysteine

123.6mg Glutamic acid

241.1mg Glutamine

9mg Glycine

65.6mg Proline

59.9mg Serine

10.9mg Tyrosine

300.2mg Arginine

182.4mg Histidine

114.1mg Isoleucine

114.1mg Leucine

158.9mg Lysine

42.5mg Methionine

47.1mg Phenylalanine

103.6mg Threonine

58.2mg Tryptophan

101.9mg Valine

Mineral Stock (per 10ml)

11mg Ferric chloride 6-hydrate

2mg Cupric chloride 4-hydrate

4mg Manganese chloride 6-hydrate

17mg Zinc sulphate

Vitamin Stock (per 5ml)

0.1mg Biotin

5mg Pantothenate

2mg Folic acid

10mg Nicotinic acid

2.5mg Pyridoxine
2.5mg Thiamine
50mg Choline
50mg Myo-inositol

Sucrose Solution (per above stock mix)
10mg Ascorbic acid
1mg Citric acid
20mg Magnesium sulphate 7-hydrate
1.7g Sucrose

Phosphate Solution (per 1ml)
150mg di-Potassium hydrogen orthophosphate 3-hydrate
(Produced as described in Prosser and Douglas, 1992).

Laboratory Techniques

All procedures adopted for the routine molecular biology experimental work were based upon standard protocols that can be found in Molecular Cloning: A Laboratory Manual (Sambrook and Russell, 2001).

Sterile Techniques

Agar was poured into sterile petri dishes (supplied by Sterilin, Waltham, USA) and allowed to cool within a 70% (v/v) ethanol treated laminar air-flow (LAF) cabinet. Set plates were spread with an ethanol flamed loop or glass rod within the proximity of a Bunsen burner. Liquefied cultures were inoculated with cells using sterile pipette tips, transferred to the media by flamed forceps within the proximity of a Bunsen burner. The necks of glass bottles and conical flasks were flamed prior to and following inoculation.

Bacterial Cell Culture

Bacterial cells were cultured using low salt LB media (LSLB), a version of the media originally described by Luria and Bertani (Bertani, 1951) containing only

0.5% (w/v) sodium chloride to maintain Zeocin antibiotic (supplied by Invivogen, San Diego, USA) activity. Media was autoclave-sterilised before use and appropriate antibiotics were added, if necessary for selection, on cooling to 50°C. Where cell colonies were required, LSLB agar was used. Agar plates were prepared and spread as described above. For analysis, 5ml aliquots of LSLB media were prepared in McCartney bottles (minipreps). Following antibiotic addition (if required), minipreps were inoculated with either an aliquot of a glycerol stock or a bacterial cell colony transferred from an agar plate as described earlier. Bacterial plates and cultures, secured on an orbital shaker set at 200rpm, were incubated overnight at 37°C. Antibiotics used in bacterial cell culturing included Ampicillin, Carbenicillin, Kanamycin and Zeocin (50µg/ml, 50µg/ml, 50µg/ml and 100µg/ml respectively).

DNA Technique

The use of nuclease-free water, sterile Eppendorf tubes and sterile pipette tips were fundamental during DNA work to ensure unwanted DNA digestion was prevented.

Plasmid DNA Isolation

Plasmid DNA was extracted from propagated cells using Wizard Plus SV Miniprep DNA Purification Systems (supplied by Promega, Madison, USA). The manufacturers instructions supplied with the kit were followed. Purified plasmid DNA was stored at -20°C.

Oligonucleotides

Oligonucleotides were synthesised and supplied by Sigma Genosys (St. Louis, USA). Primers were resuspended in nuclease-free water to a final concentration of 100µM as detailed in the manufacturer quality control sheet. Resuspended oligonucleotides were stored at -20°C.

DNA Amplification by Polymerase Chain Reaction (PCR)

To amplify DNA, standard 50µl PCR reactions were prepared in thin walled PCR tubes. These consisted of final concentrations of 0.2µM primers, 0.2mM dNTPs (supplied by Promega), 1x PCR buffer (supplied by Promega), 1.5mM $MgCl_2$, 1Unit Taq polymerase (supplied by Promega) and 1µl of DNA template. For high fidelity

PCR, proofreading Phusion polymerase (supplied by New England Biolabs, Ipswich, USA) was used. Thermo-cycling conditions were determined for each PCR to suit the type of polymerase used, the melting temperature (T_m) (as stated in the manufacturer quality control sheet) of the combination of primers and the predicted size of the PCR product (Table 2.1). Reactions were carried out using a GeneAmp PCR System 2400 thermocycler (supplied by Perkin Elmer, Waltham, USA). Amplified PCR products were visualised by DNA agarose gel electrophoresis (see page 30).

Step	Temperature	Time	
0	94°C (98°C for Phusion)	30s	
1 - Melting	94°C (98°C for Phusion)	10s-30s	
2 - Annealing	5°C lower than primer T_m	30s	x Cycles
3 - Extension	72°C	10s-3min	
4	72°C	5min	
5	4°C	∞	

Table 2.1.

Standard PCR cycling conditions for Taq polymerase (Phusion in brackets). Sections highlighted in red are the variables depending on the primers used, the length of the DNA template and amount of DNA required.

Phosphorylation and Annealing of Oligonucleotides

Oligonucleotides were phosphorylated with ATP using T4 polynucleotide kinase (supplied by Fermentas) following the manufacturer protocol. Once phosphorylated, equal amounts of the 5′ and 3′ oligonucleotides were annealed by boiling for 10 minutes and allowing them to cool naturally to room temperature.

Restriction Endonuclease Digestion of DNA

Restriction endonuclease digestion was carried out using commercially available enzymes (supplied by Fermentas, New England Biolabs and Promega). Compatible enzyme activity buffers were selected from those suggested by the manufacturers.

A typical enzyme digest:

- DNA for restriction	25µl
- 10x Enzyme activity buffer	3µl
- Required enzyme	1µl-2µl* (10Units)
	(30µl Total)

*If restriction is with two enzymes, use 1µl (10Units) for each.

Digests were placed in a heat block (supplied by Grant Instruments, Cambridge, UK) pre-set to 37°C and allowed to run to completion overnight.

DNA Agarose Gel Electrophoresis

Submarine agarose gel electrophoresis was used to separate DNA. Agarose gel solutions were produced using 1x (v/v) tris-acetic acid-EDTA buffer (TAE) with the percentage (w/v) of agarose (supplied by Bioline, London, UK) varied between 1% and 2% depending on the size of the DNA fragments to be separated. The solution was heated in the microwave until boiling clear and ethidium bromide (10mg/ml) was added at a concentration of 0.5µg/ml on cooling to 50°C, to allow visualisation on a UV transilluminator. Samples of DNA were mixed with 6x Orange G loading dye (to a final concentration of 1x) and λ DNA digested with Eco471 (supplied by Fermentas) was used as a marker of size. Gels were run at 100V, 0.05-0.1A maximum current in buffer consisting of 1x (v/v) TAE and 0.5µg/ml ethidium bromide in purified water. Complete gels were observed using a Syngene UV cabinet with Pulnix camera (supplied by Syngene, Cambridge, UK) and photographed using a Video Graphic UP-895MD printer (supplied by Sony, Minato, Japan).

Recovery of DNA from Agarose Gels

Where DNA needed to be recovered, agarose gels were visualised on a UV transilluminator (supplied by UVP inc., Upland, USA) and the required bands were cut from the gel using a sterile razor blade. Excised DNA was purified using a QIAquick Gel Extraction Kit (supplied by Qiagen, Hilden, Germany) following the manufacturer instructions. All DNA was eluted in 30µl of nuclease-free water.

Ligation of DNA

Fragments of DNA were ligated using T4 DNA ligase and ligase buffer (supplied by Promega).

A typical vector and insert ligation reaction:

- Vector DNA	4µl*
- Insert DNA	4.5µl*
- T4 Ligase reaction buffer	1µl
- T4 DNA ligase	0.5µl (1.5Units)
	(10µl Total)

*The ratio of vector DNA to insert DNA can be varied, but in the majority of cases, a slight excess (based on concentration and size) of insert DNA was used.

Ligation reactions were incubated at 4°C overnight to ensure they were complete.

Transformation of Competent *Escherichia coli* Bacterial Cells

One Shot TOP10 electro-competent *Escherichia coli* bacterial cells (supplied by Invitrogen, see page 20) were used for the expression of plasmid DNA. Cells were removed from -80°C storage, thawed upon ice and mixed with 0.5µl of the required plasmid DNA. To facilitate plasmid uptake, electroporation was carried out using a Gene Pulser system (supplied by Biorad, Hercules, USA), following recommendations by both Invitrogen (the cell provider) and Biorad (*E. coli* electroporation protocol). Once transformed, cells were mixed with 950µl LSLB media, placed on a shaker at 37°C for one hour and plated out and incubated as described on page 27. Transformants were analysed by colony PCR (see below).

Colony PCR

Colony PCR was used as a rapid method of confirming successful *E. coli* plasmid DNA transformants. Individual colonies were suspended in 10µl of sterile water by vortex mixing and boiled in a water bath for five minutes to release DNA. Cell debris was pelleted through a 30 second pulse-spin in a microcentrifuge and 5µl of the supernatant was used as a template in a PCR reaction using vector specific or gene specific primers (see page 28).

DNA Sequence Analysis

DNA sequencing was used to check constructed gene products. Appropriate vector or gene specific primers were supplied and sequencing was carried out using ABI Prism 3730 automated DNA sequencers (supplied by Applied Biosystems, Carlsbad, USA) by the DNA sequencing service (DBS Genomics), School of Biological and Biomedical Sciences, Durham University. The sequence data obtained was analysed using Sequencher software version 4.5 (Gene Codes Corporation) running on Mac OS computers.

Linearisation of Plasmid DNA

In preparation for yeast transformation, plasmid DNA was linearised. Unique restriction enzymes such as *Bln*I (supplied by Roche, Basel, Switzerland) were utilised to digest plasmid DNA from two minipreps (see page 28).
A standard linearisation reaction:

- Plasmid DNA for linearisation	80µl
- 10x enzyme activity buffer	9µl
- Unique cutting enzyme	1µl-2µl (10Units)
	(90µl Total)

Reactions were incubated similarly to standard restriction endonuclease reactions.

Extraction and Precipitation of DNA

Solutions of DNA were cleaned up via a phenol-chloroform extraction. An equal volume of phenol-chloroform was added to the sample. The mixture was vortexed for two minutes and subjected to centrifugation in a microcentrifuge at maximum speed for 10 minutes. The top layer aqueous phase was carefully removed and put through another round of extraction with just chloroform. An equal volume of chloroform was added to the sample. Again, the mixture was vortexed for two minutes and subjected to centrifugation in a microcentrifuge at maximum speed for 10 minutes. The top layer aqueous phase was carefully removed for ethanol precipitation. The volume of the extracted DNA solution was measured and one tenth volume of 3M sodium acetate, pH 5.2 was added. Two volumes of chilled ethanol was added and the mixture was briefly vortexed before incubation overnight at -20°C. Centrifugation at 11000x g (12500rpm), 4°C for 15 minutes (Beckman

rotor F2402) pelleted the precipitated DNA. The supernatant was carefully removed and 1ml of 75% (v/v) ethanol was added prior to vortexing to resuspend and wash the DNA pellet. Centrifugation at 11000x g (12500rpm), 4°C for 10 minutes (Beckman rotor F2402) again pelleted the DNA. With the supernatant carefully removed, the precipitated DNA pellet was vacuum dried in a dessicator (supplied by Jencons, Bridgeville, USA) and resuspended in 5µl of nuclease-free water.

Transformation of Competent *Pichia pastoris* Yeast Cells

Easycomp SMD1168H *Pichia pastoris* yeast cells (supplied by Invitrogen, see page 20) were used for the expression of gene constructs. Cells were removed from -80°C storage and thawed on ice. Heat-shock transformation was carried out using the Easycomp *Pichia pastoris* kit following the manufacturer instructions (Invitrogen). Following four days growth, transformants were propagated and analysed by western blotting (see page 37).

Yeast Cell Culture

Yeast cells were cultured using yeast extract-peptone-glycerol media (YPG). Similarly to bacterial cell culture, YPG media was autoclave-sterilised before use and appropriate antibiotics were added, if necessary for selection, on cooling to 50°C. Where cell colonies were required, YPG agar was used. Agar plates were prepared and spread as described on page 27. For analysis, 10ml aliquots of YPG media were prepared in McCartney bottles. Following antibiotic addition (if required), YPG was inoculated with either an aliquot of a glycerol stock or a yeast cell colony transferred from an agar plate, as documented earlier. Yeast plates and cultures, secured on an orbital shaker set at 250rpm, were incubated for four days at 30°C. The main antibiotic used in yeast cell culturing was Zeocin (100µg/ml).

Bacterial and Yeast Glycerol Stocks

Glycerol stocks were prepared to maintain a supply of competent cells successfully transformed with modified DNA plasmids. Bacterial cells were propagated in LSLB media overnight and yeast cells were grown in YPG media over four days. A 750µl aliquot of the resultant cell culture was mixed with 250µl of sterile 60% (v/v) glycerol in sterile 1.5ml cryogenic tubes (supplied by Sarstedt, Germany). Cryogenic tubes were snap frozen in liquid nitrogen and stored at -80°C.

Large Scale Yeast Cell Culture (Fermentation)

BioFlo 110 7.5-litre autoclavable bench-top fermenters (supplied by New Brunswick Scientific, Enfield, USA) were used to produce cultures for protein purification. A basal three litres of autoclave-sterilised minimal media (Higgins and Cregg, 1998) supplemented with PTM1 trace salts (Cino, 1999) was inoculated with three 100ml YPG seed cultures (grown for three days). Cultivation limits were set at 30°C, pH 4.0-5.0 and 30% dissolved oxygen with continuous agitation. A glycerol feed of 5-10ml/hour was maintained over three days. The pGAPZα expression vectors used (supplied by Invitrogen) were specifically chosen as they contained the yeast alpha factor (extracellular secretion signal), hence, recombinant protein was secreted into the surrounding media. Cells were pelleted by centrifugation for 25 minutes at 5500x g (7000rpm) and 4°C (Beckman rotor JLA10.500) so that the supernatant could be processed further (see below).

Chromatography and Purification of Recombinant Proteins

Yeast culture supernatant containing recombinant protein, was filtered through 2.7µm GF/D and 0.7µm GF/F glass microfibre filter discs (supplied by Whatman Ltd., Little Chalfont, UK) in succession using a vacuum manifold. Based on the recombinant protein properties (pI and purification tags etc.), the supernatant was subject to ion-exchange chromatography (SP Sepharose or Phenyl Sepharose, supplied by GE Healthcare, Little Chalfont, UK) or metal affinity chromatography (Ni-NTA HisTrap FF, supplied by GE Healthcare). Chromatography columns (supplied by GE Healthcare) were sanitised and rinsed prior to use, following the manufacturer recommendations. Manufacturer recommended buffers were also used (listed on page 24). Loading supernatants (2ml/minute) were allowed to re-circulate overnight at 4°C to encourage maximum protein binding (room temperature for Ni-NTA HisTrap FF columns). For hydrophobic interaction chromatography elution, increasing or decreasing linear gradients of sodium chloride concentration were generated using a fast protein liquid chromatography (FPLC) system (supplied by GE Healthcare). Throughout FPLC system purifications, 5ml fractions were collected via an attached fraction collector. The fractions showing an increased UV absorbance at a wavelength of 280nm were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (see next page) to establish which contained the recombinant protein. Nickel column elution peaks were collected as a

single fraction and analysed by SDS-PAGE. To maximise recombinant protein recovery from large volumes of supernatant, the column flow-through was reprocessed at least twice. Partially purified recombinant proteins were manually run through a gel filtration column (Sephacryl S-200 matrix, supplied by GE Healthcare) at 0.5ml/minute to size exclude high molecular weight yeast proteins. The column was sanitised and rinsed prior to use, following the manufacturer recommendations. Gel filtration was carried out in 20mM diaminopropane (DAP), 0.1M phosphate buffered saline (PBS), pH 9.0. Throughout the separation, 2.5ml fractions were collected and those showing an increased UV absorbance at a wavelength of 280nm were analysed using SDS-PAGE.

Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis with the discontinuous pH system originally described by Laemmli (1970) was used to separate denatured proteins. Standard gels with 12 wells were cast using 1mm depth gel plate kits (supplied by ATTO Corporation, Tokyo, Japan) following the Qiagen SDS-PAGE protocol. The percentage (v/v) of acrylamide solution (supplied by National Diagnostics) contained within the resolving gel varied between 12.5% and 20% depending on the size of the proteins to be separated. Protein samples were mixed with 5x SDS loading dye (to a final concentration of 1x) and boiled alongside SDS 7 molecular weight marker (supplied by Sigma) in a water bath for 10 minutes, to allow protein denaturation and reduction prior to loading. Gels were run at 100V, 0.05-0.1A maximum current through the stacking gel and 200V, 0.05-0.1A maximum current through the resolving gel in gel tanks (supplied by ATTO Corporation) filled with 1x reservoir buffer. Complete gels were either Coomassie stained, silver stained or prepared for western blotting (see page 37). Coomassie staining was carried out for three hours followed by de-staining for analysis. Both staining and de-staining were carried out with gentle agitation at room temperature. Silver staining was carried out using a modified version of the protocol described by Blum *et al.* (1987). Gels underwent a one hour incubation in fixative solution, two 20 minute washes in wash solution and a single wash in distilled water for 20 minutes. This was followed by a one minute incubation in sensitizer solution, three 20 second distilled water washes, a 20 minute incubation in silver nitrate solution and a further

three 20 second washes in distilled water. Gels were incubated in developing solution until proteins were resolved sufficiently. Finally, gels were washed for 20 seconds in distilled water and incubated in stop solution for five minutes. All gels were scanned into a Mac OS computer and captured as JPEG files.

Dialysis and Freeze Drying of Protein Samples

Chromatography column eluates and protein solutions were buffer exchanged into purified water via dialysis. Dialysis tubing (supplied by Medicell International Ltd., London, UK) with the correct molecular weight cut off for the protein was prepared by boiling for 20 minutes in purified water containing 5mM ammonium hydrogen carbonate and a trace of ethylenediaminetetra-acetic acid (EDTA). Rinsed tubing containing protein sample was double clamped and placed in at least 100x greater volume of purified water. Dialysis was carried out at 4°C with constant stirring and water replaced at least twice in 24 hours, ensuring initial buffer concentration was reduced to less than 50nM. Unless stated otherwise, dialysed protein samples were frozen on the walls of round-bottomed flasks by shelling under liquid nitrogen, lyophilised by an FTS System Flexi Dry MP freeze-drier and stored at 4°C. For solution volumes of less than 5ml, Slide-A-Lyzer dialysis cassettes were used (supplied by Thermo Scientific, Waltham, USA). Apart from being filled by using a syringe, the dialysis procedure was identical to that described above.

Estimation of Protein Concentration

Where possible, recombinant protein concentration was estimated by SDS-PAGE (see page 35). Protein samples were run alongside known concentrations of a suitable commercial protein standard and stained as described previously. However, for samples containing many different proteins, a bicinchoninic acid assay (BCA) (reagents supplied by Thermo Scientific) was carried out, using bovine serum albumin (BSA) in PBS as the standard protein, following the manufacturer instructions. Prepared 96 well microtitre plates were absorbance measured using a VersaMax microplate reader (supplied by Molecular Devices, Sunnyvale, USA) with BCA assay settings pre-programmed.

Western Blotting

Western blotting was used as a more sensitive approach for detecting proteins. The semi-dry electro-transfer method documented thoroughly by Kurien and Scofield (2006) was used in combination with chemilluminescent detection of proteins, carried out following the Qiagen protein analysis benchguide. Samples for analysis were run on SDS-PAGE gels (described on page 35), which were subsequently equilibrated for five minutes in Bjerrum and Schafer-Nielsen buffer (Bjerrum and Schafer-Nielsen, 1986). Optitran BA-S 85 reinforced nitrocelluose membrane and 3MM blotting papers (both supplied by Whatman Ltd.) were cut to match the dimensions of the gel and pre-soaked similarly in Bjerrum and Schafer-Nielsen buffer. Blotting was carried out using HorizBlot electroblotting systems (supplied by ATTO Corporation) with a constant current of 0.15A ($2mA/cm^2$) for a minimum of one hour as per the Qiagen protocol. Transfer was confirmed by using Ponceau S stain to stain the membrane and molecular weight markers prior to destaining with distilled water. Fresh solutions of antisera block and rinse were produced for every blot and membrane incubations were again carried out following the Qiagen protein analysis benchguide. Western blots were probed with appropriate specific primary antibodies, including monoclonal anti-avidin (supplied by Sigma, used at 1:10000 dilution), anti-His-tag (supplied by Novagen, Darmstadt, Germany, used at 1:1000 dilution) and monoclonal anti-GNA (raised in rabbit, used at 1:3000 dilution) and were allowed to incubate overnight with gentle agitation. Peroxidase-coupled secondary antibodies (anti-rabbit or anti-mouse) (supplied by Thermo Scientific, used at 1:5000 dilution) were left to bind for a minimum of one hour with gentle agitation. Enhanced chemilluminsecent (ECL) reagents (supplied by Sigma) were used to detect specifically bound secondary antibodies by adding 5ml of ECL solution to the membrane prior to exposure to X-Ray film (supplied by Fujifilm Ltd., Tokyo, Japan) in a lead-lined film cassette. Films were developed using an automatic Compact X4 developer (supplied by X-ograph Imaging systems, Stonehouse, UK).

Protein Deglycosylation

Proteins were deglycosylated with Peptide: N-Glycosidase F (PNGase F) (supplied by New England Biolabs) as recommended by the manufacturer. Highly glycosylated yeast invertase was included as a positive control. Reaction products were analysed by SDS-PAGE (see page 35).

Avidin *in vitro* Functionality Assay

The functionality of avidin was assayed by its ability to bind biotin *in vitro* using biotin-agarose (supplied by Thermo Scientific). Following the manufacturer recommendations, matrix was incubated with 5μg of protein at room temperature for 30 minutes with agitation. After microcentrifugation for one minute at 3000rpm, the pellet was washed twice with PBS. Avidin bound to the biotin of the matrix was eluted by boiling, and unbound, wash and elution fractions were analysed by SDS-PAGE (see page 35).

Fluorescent Labelling of Proteins

Recombinant proteins were fluorescently labelled with fluorescein isothiocyanate (FITC). This labels amine and sulphydryl groups of proteins. Dimethyl sulphoxide (DMSO) was used to dissolve FITC as recommended by the manufacturer (Sigma). Proteins were resuspended in 50mM carbonate, pH 9.0 and mixed with a 2:1 molar excess of FITC, to ensure complete saturation with the fluorescent label. The reaction was allowed to proceed for three hours at room temperature with gentle agitation, covered in foil to prevent photobleaching. Following incubation, the excess unbound FITC was removed by dialysis against purified water (see page 36), again covered in foil. Labelled proteins were stored at -20°C.

Microscopy

For all dissections, a Vision SX45 stereo zoom microscope (supplied by Vision Engineering, Woking, UK) was used with 10x eyepiece lenses. For fluorescence microscopy, a Nikon Eclipse TE300 fluorescent microscope was used. Images were captured using an attached Hamamatsu camera and Openlab software version 4 (Perkin Elmer) on a Mac OS computer.

N-Terminal Protein Sequencing

Protein N-terminal sequencing was carried out by Cambridge Peptides, Philip Victor Road, Birmingham, West Midlands, UK. Samples were prepared following instructions supplied by Cambridge Peptides.

Protein Biotinylation

Biotinylation of proteins was carried out using the Protein Biotinylation Module (supplied by GE Healthcare) following the manufacturer instructions. Proteins were initially re-suspended in sodium bicarbonate as recommended. Following biotinylation, proteins were dialysed into PBS as described on page 36. Biotinylated proteins were stored at -20°C. More complex biotinylated peptides were synthesised by Cambridge Research Biochemicals, Billingham, Cleveland, UK.

Conjugation of Biotinylated Peptides to Avidin

Conjugation was simply carried out by allowing avidin to mix with the biotinylated peptide. Avidin was resuspended in PBS and mixed with a 2:1 molar excess of biotinylated peptide (see above) to ensure complete saturation of the biotin binding sites. The conjugation was allowed to proceed for three hours at room temperature with constant agitation. Following incubation, the excess unbound biotinylated peptide was removed by dialysis against purified water (see page 36). Avidin conjugates were stored at -20°C.

Insect Bioassays and Dissection Techniques

Hemipteran Artificial Diet Feeding Bioassays

Adult aphids were selected from plants 48 hours before starting the feeding bioassay and placed in Perspex aphid feeding chamber rings (40mm diameter) covered by two sheets of parafilm between which, 100µl of artificial diet (Prosser and Douglas, 1992, see page 26) was sandwiched (Figure 2.1). Aphids were left to produce nymphs overnight. The following day, the adults were removed and the nymphs were maintained on the artificial diet for a further 24 hours. Aphid feeding chambers were prepared with treatment diets immediately before starting the bioassay. A total of 100µl of solution was used for each feeding chamber, comprising of 75µl of artificial diet and 25µl of test protein at the required concentration. Bioassays were carried out in duplicate, with 20 aphids transferred to each treatment (10 per chamber) and their survival and growth was monitored daily for 10 days, with the diet refreshed as required. After 10 days, if survival was high, aphids were imaged using an Olympus SZH10 stereo microscope with a QICAM

camera attached. Images were captured using Openlab software version 4 (Perkin Elmer) and analysed using ImageJ software version 1.41 (National Institutes of Health, USA), measuring the overall length and width of the aphids.

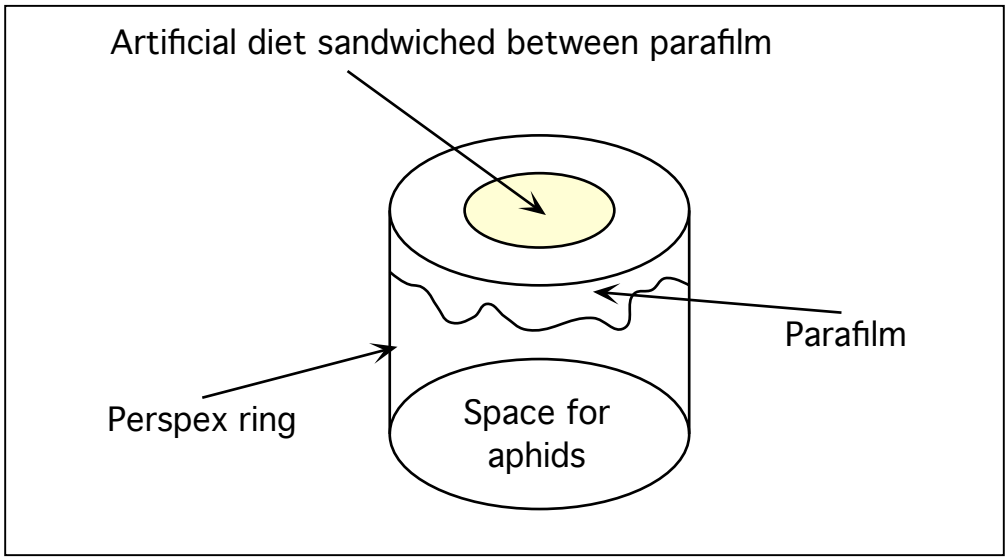


Figure 2.1.
Schematic representation of an aphid feeding chamber.

Hemipteran Whole Insect Extracts

Forty whole aphids were frozen rapidly in liquid nitrogen, added to 200µl of 50mM HEPES, 0.1% (v/v) Triton X100, pH 7.4 and hand-homogenised using an autoclave-sterilised pestle. Debris was pelleted by microcentrifugation at maximum speed and the supernatant extracted material removed for analysis or assay use. The total protein content of the aphid extracts was estimated by BCA assay and SDS-PAGE (see pages 36 and 35).

Hemipteran Haemolymph and Gut Extraction

Haemolymph was extracted from aphids by carefully removing a front leg and placing a glass capillary to the wound to collect extruded material. Collected haemolymph was added directly to 5x SDS loading dye in preparation for SDS-PAGE and western blotting (see pages 35 and 37). Guts were removed under purified water by carefully pulling the head from the aphid using fine forceps and allowing the attached gut to follow. The head was removed once the gut was completely

pulled free of the body. Extracted guts were either observed by fluorescence microscopy (see page 38) or prepared for further analysis. For analysis, guts were collected in 100µl of purified water and homogenised manually using an autoclave-sterilised pestle. Debris was pelleted by microcentrifugation at maximum speed and the supernatant gut material was removed for total protein quantitation and preparation in 5x SDS loading dye for SDS-PAGE and western blotting (see pages 35 and 37).

Amplification of Genomic Region Containing Biotin Synthesis Genes from Hemipteran *Buchnera* Genomic DNA

DNA was extracted from whole adult aphids using TRI reagent (supplied by Sigma) according to the manufacturer instructions. Oligonucleotide primers for PCR were designed based on the published *A. pisum Buchnera* genome sequence (http://buchnera.gsc.riken.go.jp), using sequences in flanking *glyA* and *ybhE* genes to amplify the region containing genes encoding biotin synthesis enzymes (primers listed in Table 2.2). Experiments using *A. pisum* DNA as a positive control showed that a product of the correct size was amplified. This was cloned into pJET (supplied by Clontech, Shiga, Japan) and DNA sequencing was used to confirm that it corresponded to the expected fragment. Amplification of DNA extracted from *S. avenae* gave a fragment of similar size to that obtained from *A. pisum*. This was cloned as detailed above and analysed by DNA sequencing, using a 'primer walking' method to obtain the complete sequence of the fragment.

Primer	Sequence (5′ → 3′)
glyA 5′	CAAGGTGGACCATTAATGCAT
ybhE 3′	GGTCCGCGTCATATCATCTTTCATCC
Round 2 5′	AATTTACTTAAGATATGAGGTGGAGC
Round 2 3′	CAATATGACGTGAAGTTAGAGTTGC
Round 3 5′	CGCCTAAACCTATGATTCCTCCAG

Table 2.2.
Oligonucleotide primers used to amplify and sequence the biotin operon.

Hemipteran Gut Pull-Down Assay

- Magnetic Bead Preparation

Magnabind amine-derivatized magnetic beads (supplied by Thermo Scientific) were cross-linked to recombinant avidin using a *Bis*[sulfosuccinimidyl] suberate (BS^3) cross-linker (supplied by Thermo Scientific) following the manufacturer instructions. The coupling efficiency was determined from an initial UV absorbance at a wavelength of 280nm and a value for the supernatant after the cross-linking reaction had been stopped. It was calculated that 91% of the recombinant avidin had been cross-linked to the beads, giving a final bead solution concentration of 9.1mg/ml recombinant avidin.

- Gut Sample Preparation

Approximately one hundred adult *A. pisum* guts were dissected under purified water and placed in a chilled eppendorf tube containing 100µl of PBS. The guts were homogenised manually using an autoclave-sterilised pestle and debris was pelleted by microcentrifugation at 12000rpm, 4°C for 10 minutes. The supernatant gut protein solution was stored at -20°C until required.

-Pull-Down Assay

Gut samples were thawed, mixed with 50µl of recombinant avidin magnetic beads and made up to a final volume of 0.5ml with PBS. The mixture was placed on a roller at 4°C overnight. The beads were collected using a magnetic stand and the supernatant removed and kept as the unbound fraction. The beads were washed for five minutes with 0.5ml of PBS. This was repeated three times, each time collecting the beads and keeping the supernatant as wash fractions. After removal of the final wash fraction, the beads were resuspended in 1x SDS-PAGE loading dye and this was the elution fraction. Aliquots of 25µl of the starting gut sample preparation, the unbound fraction, the wash fractions and a sample of the magnetic beads were prepared for SDS-PAGE alongside the elution fraction. Prior to loading, samples were spun briefly in a microcentrifuge to pellet and prevent the magnetic beads being loaded into the wells of the gel. Due to the small amounts of protein, the gel was silver stained.

Lepidopteran Haemolymph, Gut Contents, Gut and Nerve cord Extraction

Haemolymph, gut contents, gut and nerve cord samples from lepidopteran larvae were extracted and processed as described in Fitches *et al.* (2001) with the exceptions that intact guts were flushed with purified water and the extraction buffer was PBS containing 1mM dithiothreitol (DTT) (Figure 2.2). The total protein concentration of the gut contents and gut-processed supernatants were estimated by BCA assay (see page 36). Haemolymph, nerve cord and equal amounts of quantified gut samples were analysed for the presence of the required proteins by western blotting (see page 37).

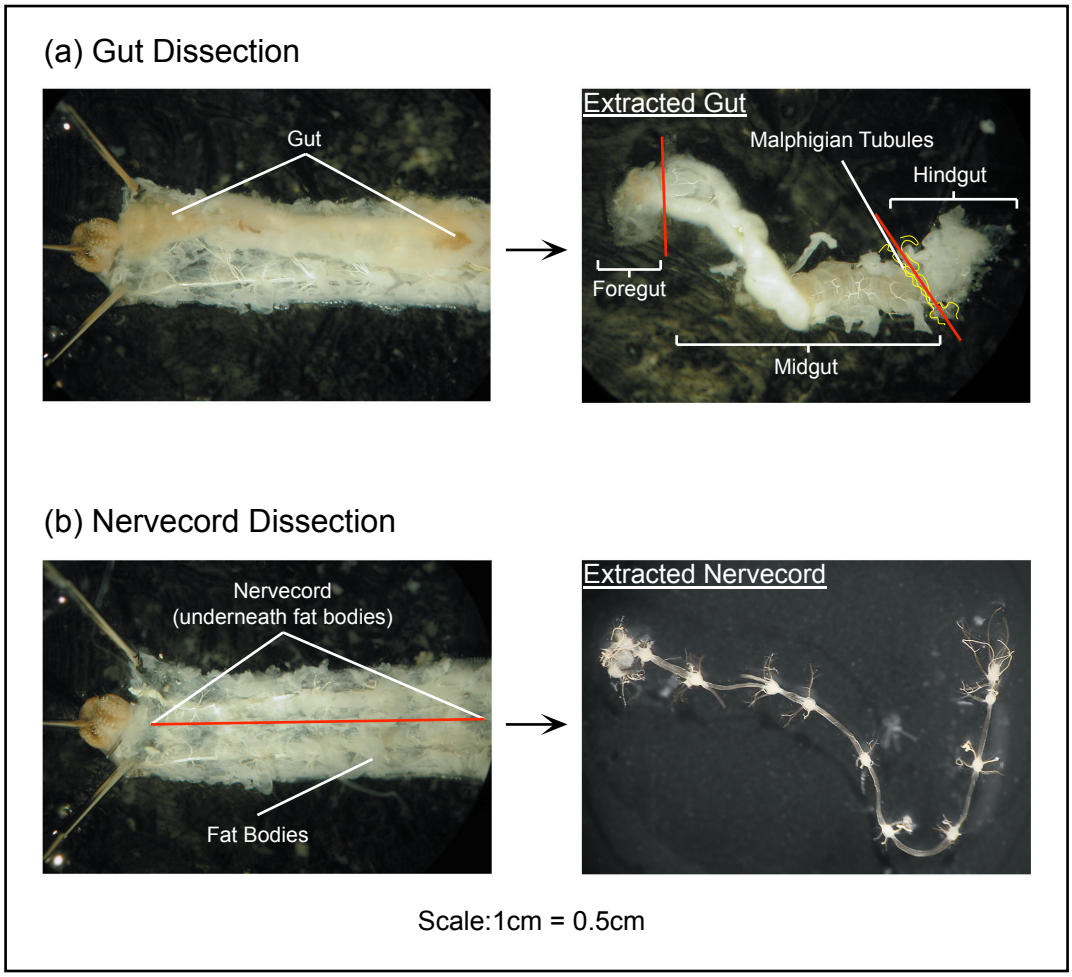


Figure 2.2.

Micrographs of *M. brassicae* larvae dissection.
(a) Gut dissection. (b) Nerve cord dissection. Labels define the dissected components.

Lepidopteran Injection Bioassays

Purified recombinant proteins were tested for their biological activity by injecting day one, fifth stadium *M. brassicae* larvae of similar size with a maximum of 100µg of protein (quantified by SDS-PAGE) re-suspended in 5µl of PBS. Larvae were anaesthetised with carbon dioxide for 30 seconds and injected behind the head capsule using a 10µl Hamilton syringe (supplied by Scientific Laboratory Supplies, Hessle, UK). Unless otherwise stated, batches of 10 larvae were injected for each treatment concentration, alongside 10 others injected with equivalent amounts of PBS as a negative control (previously shown to have no effect). Following injection, the larvae were maintained on artificial diet in clear plastic pots (five larvae per pot) and monitored over the following days until pupation (if required). Fresh artificial diet was added to the pots as necessary. For *in vivo* analysis of protein transport, larvae were dissected after the required amount of time had passed, as described on page 43.

Lepidopteran Droplet Feeding Bioassays

Purified recombinant proteins were tested for their oral activity by feeding a 5µl droplet containing a maximum of 50µg of protein with 0.5% (w/v) sucrose (as a 'sweetener') to newly moulted fifth stadium *M. brassicae* larvae. Larvae had been starved for 24 hours prior to the bioassay. Unless otherwise stated, batches of 10 larvae were droplet fed for each treatment concentration, alongside 10 others droplet fed with equivalent amounts of PBS as a negative control (previously shown to have no effect). Larvae were also maintained, monitored or dissected.

Lepidopteran Artificial Diet Feeding Bioassays

Conjugates were tested for their oral activity by inclusion in a powdered optimal artificial diet (supplied by Bio-Serv, Frenchtown, USA). Protein was added to 5g wet weight of diet at the required concentration and set with 1% (w/v) agar. Neonate *M. brassicae* larvae were carefully placed on the diet in clear plastic pots (10 larvae per pot) and monitored over the following days. Unless otherwise stated, batches of 10 larvae were fed for each treatment concentration, alongside 10 others fed with optimal artificial diet as a negative control. For *in vivo* analysis of conjugate transport, haemolymph was extracted after the required amount of time had passed, as described on page 43.

Production of Recombinant Fusion Proteins

ButaIT-Avidin 1 (pGAPZαB)

The ButaIT toxin coding sequence was PCR-amplified with 5′ *Pst*I and 3′ *Sal*I restriction end sites (primers listed in Table 2.3) using the ButaIT-GNA pGAPZαB expression construct (Pham-Trung, 2006) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 10 seconds, 60°C 30 seconds, 72°C 10 seconds, 25 cycles). After purification by agarose gel electrophoresis and extraction, the PCR product was restricted with *Pst*I and *Sal*I. The product was subsequently ligated into the recombinant avidin pGAPZαB expression construct (Hinchliffe *et al.*, 2010), which had also been restricted with *Pst*I and *Sal*I. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

ButaIT-Avidin 2 (pGAPZαB)

The Avidin coding sequence was PCR-amplified with 5′ *Not*I and 3′ *Xba*I restriction end sites (primers listed in Table 2.3) using the recombinant avidin pGAPZαB expression construct (Hinchliffe *et al.*, 2010) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 15 seconds, 57°C 30 seconds, 72°C 20 seconds, 25 cycles). The GNA lectin coding sequence was restricted from the ButaIT-GNA pGAPZαB expression construct (Pham-Trung, 2006) using *Not*I and *Xba*I. Following purification by agarose gel electrophoresis and extraction, the avidin PCR product was restricted with *Not*I and *Xba*I. The product was subsequently ligated in place of the removed GNA lectin coding sequence described above. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

Avidin-ButaIT 3 (pGAPZαB)

In a similar manner to ButaIT-Avidin 2, the avidin coding sequence was PCR-amplified with 5′ *Pst*I and 3′ *Not*I restriction end sites (primers listed in Table 2.3) using the recombinant avidin pGAPZαB expression construct (Hinchliffe *et al.*, 2010) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 15 seconds, 65°C 30 seconds, 72°C 20 seconds, 25 cycles). The GNA lectin coding sequence was restricted from the GNA-ButaIT pGAPZαB expression construct (Fitches *et al.*, 2010) using *Pst*I and *Not*I. After purification by agarose gel electrophoresis and extraction, the avidin PCR product was restricted with *Pst*I and *Not*I. The product was subsequently ligated in place of the removed GNA lectin coding sequence described above. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT (pGAPZαB)

Oligonucleotide primers with *Not*I restriction end sites were designed to incorporate the Immunoglobulin G (IgG) hinge coding sequence (Airenne and Kulomaa, 1995) (primers listed in Table 2.3). The IgG hinge primers were phosphorylated, annealed and ligated into both the ButaIT-Avidin 2 and Avidin-ButaIT 3 pGAPZαB expression constructs (produced as described above), which had been restricted with *Not*I. As both restriction end sites were *Not*I (due to the absence of alternative restriction sites), it was expected that a proportion of the hinge insert would ligate in the correct orientation, while the remainder would ligate in the reverse direction. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

ωACTXHv1a-Avidin (Omega Atracotoxin) (pGAPZαB)

The ωACTXHv1a-Avidin fusion protein expression construct was produced utilising the existing ButaIT-Avidin 2 pGAPZαB (produced as described on page 45) and ωACTXHv1a-GNA pGAPZαB (supplied by Dr Elaine Fitches) expression

constructs. The ButaIT toxin coding sequence was restricted from the ButaIT-Avidin 2 pGAPZαB expression construct using *Pst*I and *Not*I and the ωACTXHv1a toxin coding sequence was restricted from the ωACTXHv1a-GNA pGAPZαB expression construct similarly with *Pst*I and *Not*I. Following purification by agarose gel electrophoresis and extraction, the ωACTXHv1a toxin coding sequence was ligated in place of the removed ButaIT toxin coding sequence described above. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

eGFP-Avidin (enhanced Green Fluorescent Protein) (pGAPZαB)

The eGFP chromophore coding sequence was PCR-amplified with 5′ *Pst*I and 3′ *Not*I restriction end sites (primers listed in Table 2.3) using the eGFP pGAPZαB expression construct (supplied by Dr Daniel Price, Accession Number HQ259114.1) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds, 25 cycles). The ButaIT toxin coding sequence was restricted from the ButaIT-Avidin 2 pGAPZαB expression construct (produced as described on page 45) using *Pst*I and *Not*I. After purification by agarose gel electrophoresis and extraction, the eGFP PCR product was restricted with *Pst*I and *Not*I. The product was subsequently ligated in place of the removed ButaIT toxin coding sequence described above. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

ButaIT-GNA Fragment-Avidin (ButaIT-Gavidin) (pGAPZαB)

The ButaIT-GNA Fragment coding sequence was PCR-amplified with 5′ *Pst*I and 3′ *Sal*I restriction end sites (primers listed in Table 2.3) using the ButaIT-GNA pGAPZαB expression construct (Pham-Trung, 2006) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 15 seconds, 66°C 30 seconds, 72°C 15 seconds, 25 cycles). The ButaIT toxin coding sequence was restricted from the ButaIT-Avidin 1 pGAPZαB expression construct (produced as described on page

45) using *Pst*I and *Sal*I. Following purification by agarose gel electrophoresis and extraction, the ButaIT-GNA Fragment PCR product was restricted with *Pst*I and *Sal*I. The product was subsequently ligated in place of the removed ButaIT toxin coding sequence described above. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

ButaIT-Gavidin (1 Mutation) (pGAPZαB)

End-to-end oligonucleotide primers were designed to allow PCR amplification around the complete plasmid of the ButaIT-Gavidin pGAPZαB expression construct (produced as described above) (primers listed in Table 2.3) (Phusion polymerase, PCR thermocycling conditions: 98°C 30 seconds, 59°C 30 seconds, 72°C 2 minutes 40 seconds, 25 cycles). The 5′ primer incorporated a double nucleotide base change mutation (underlined in Table 2.3). Following amplification around the plasmid, the blunt ends were phosphorylated and ligated as described on page 29. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct mutation of the expression construct.

ButaIT-Gavidin (3 Mutations) (pGAPZαB)

Similarly to ButaIT-Gavidin 1 Mutation, end-to-end oligonucleotide primers were designed to allow PCR amplification around the complete plasmid of the ButaIT-Gavidin pGAPZαB expression construct (produced as described on page 47) (primers listed in Table 2.3) (Phusion polymerase, PCR thermocycling conditions: 98°C 30 seconds, 68°C 30 seconds, 72°C 2 minutes 40 seconds, 25 cycles). Both the 5′ primer and the 3′ primer incorporated two double nucleotide base change mutations (underlined in Table 2.3). Following amplification around the plasmid, the blunt ends were phosphorylated and ligated as described on page 29. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified

DNA was sequenced to confirm the presence and correct mutations of the expression construct.

ButaIT-GNA Fragment (pGAPZαB)

The ButaIT-GNA Fragment fusion protein expression construct was produced utilising the existing ButaIT-Gavidin pGAPZαB expression construct (produced as described on page 47). The ButaIT-GNA Fragment coding sequence was restricted from the ButaIT-Gavidin pGAPZαB expression construct using *Pst*I and *Sal*I. After purification by agarose gel electrophoresis and extraction, the ButaIT-GNA Fragment coding sequence was ligated into pGAPZαB vector that had also been restricted with *Pst*I and *Sal*I. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

GNA Fragment-Avidin (pGAPZαB)

The GNA Fragment-Avidin coding sequence was PCR-amplified with 5′ *Pst*I and 3′ *Not*I restriction end sites (primers listed in Table 2.3) using the ButaIT-Gavidin pGAPZαB expression construct (produced as described on page 47) as a template (Phusion polymerase, PCR thermocycling conditions: 98°C 20 seconds, 62°C 30 seconds, 72°C 20 seconds, 25 cycles). Following purification by agarose gel electrophoresis and extraction, the PCR product was restricted with *Pst*I and *Not*I. The product was subsequently ligated into pGAPZαB vector that had also been restricted with *Pst*I and *Not*I. The ligation mixture was transformed into *E. coli* and the resultant clones were screened by colony PCR. Recombinant plasmid was extracted from positive clones and purified DNA was sequenced to confirm the presence and correct assembly of the expression construct.

Primer	Sequence (5′ → 3′)	Enzyme site
ButaIT-Avidin 1 5′	TAACTGCAGCAAGGTGTGGTCCTTGCTTT	*Pst*I
ButaIT-Avidin 1 3′	TTAGTCGACTTGTATACCACAGATACATTG	*Sal*I
ButaIT-Avidin 2 5′	TAAGCGGCCGCCGCTAGAAAATGCTCG	*Not*I
ButaIT-Avidin 2 3′	TTATCTAGATCATTCCTTTTGAGTTCTC	*Xba*I
Avidin-ButaIT 3 5′	TAACTGCAGCTAGAAAATGCTCGCTG	*Pst*I
Avidin-ButaIT 3 3′	TTAGCGGCCGCTTCCTTTTGAGTTCTCAA	*Not*I
IgG Hinge 5′	GGCCGCACCAAAGCCAAGTACTCCACCAGGTAGTAGTGC	*Not*I
IgG Hinge 3′	CGTGGTTTCGGTTCATGAGGTGGTCCATCATCACGCCGG	*Not*I
eGFP-Avidin 5′	TAACTGCAGCAATGGTGAGCAAGGGC	*Pst*I
eGFP-Avidin 3′	GCGGCGGCCGCCTTGTACAGCTCGTC	*Not*I
ButaIT-Gavidin 5′	TAACTGCAGCAAGGTGTGGTCCTTGCTTT	*Pst*I
ButaIT-Gavidin 3′	TTAGTCGACGTTGAGAAATTCCCCTGTAGAGAG	*Sal*I
1 Mutation 5′	ACTGCCTCTACAGGGGAATTTCTC	-
1 Mutation 3′	CTCACCGGAGTACAAAATATTGTC	-
3 Mutations 5′	ACTGCCTCTACAGGGGAATTTGCCAACGTCGACGCT	-
3 Mutations 3′	CTCACCGGAGGTCGCAATATTGTCGGCGGCCGC	-
GNA Frag-Avidin 5′	TTACTGCAGCCGACAATATTTTGTAC	*Pst*I
GNA Frag-Avidin 3′	TAAGCGGCCGCTCAATGATGATGATGATGATGGTCGAT	*Not*I

Table 2.3.

Oligonucleotide primers used to produce avidin fusion proteins.
Enzyme or mutation sites are underlined.

Statistical Analysis

All data analysis was conducted using the statistical functions of Prism version 5.0 software (GraphPad Software, Inc.). Kaplan-Meier insect survival curves were compared using Mantel-Cox log-rank tests, again using Prism software.

Chapter 3

Comparison of Recombinant Avidin with Commercially Available Avidin

Introduction

A previous study with recombinant avidin produced in *Pichia pastoris* was directed towards analysing its transport from the gut to the haemolymph *in vivo* and its toxicity towards lepidopteran insects (Hinchliffe, 2007). The insecticidal activity of recombinant avidin was demonstrated in *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae together with its transport to the haemolymph following ingestion. However, little consideration was given to the scientific basis of how recombinant avidin compared to the Sigma commercial alternative (product number: A9275-25MG from accession number: CAC34569). The initial experiments of this chapter therefore focus on the characterisation of recombinant avidin.

The difference between recombinant avidin and the commercial avidin is a nine amino acid N-terminal extension and a C-terminal $(His)_6$ tag (Appendix 1). The *P. pastoris* yeast expression host is documented to carry out glycosylation and sometimes hyperglycosylation (Cregg *et al.*, 1993). The difference in the amount of glycosylation will be evident by the change in size observed on sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) gel following deglycosylation with PNGase F.

From the amino acid sequence (Appendix 1), it is predicted that recombinant avidin will form tetramers similar to commercial avidin. To see if this is correct, gel filtration of a sample of both will be carried out. Finally, to ensure that recombinant avidin is functional, immobilised biotin will be bound, rinsed and analysed on SDS-PAGE gel.

The scientific literature contains no published reports of the insecticidal effects of avidin towards hemiptera. Aphids are important globally as pests of crops, not only for the direct damage they cause by infestation, but also through their role as vectors of plant diseases (Chrispeels and Sadava, 2003). The later experiments of this chapter therefore focus around the effects and differences of recombinant avidin upon two species of aphid: *Acyrthosiphon pisum* (pea aphid) (Hemiptera: Aphididae, Harris 1776) and *Sitobion avenae* (cereal aphid) (Hemiptera: Aphididae, Fabricius 1775).

The final experiments of this chapter look again at the *in vivo* transport of recombinant avidin from the gut to the haemolymph of insects, making comparisons between hemiptera and lepidoptera, which form the basis of the experimental design later in this study (Chapters 4 and 5).

Results

For these experiments, commercial egg-white avidin was supplied by Sigma (St. Louis, USA) (product number: A9275-25MG). Recombinant avidin was previously produced in *P. pastoris* and purified by nickel affinity chromatography, as described in Hinchliffe (2007). The nucleotide and amino acid sequence of recombinant avidin can be found in Appendix 1, or Hinchliffe *et al.*, 2010 (Appendix 2).

Characterisation of Recombinant Avidin

1. Deglycosylation

Deglycosylation *in vitro* showed that recombinant avidin contained covalently linked carbohydrate, indicating glycosylation by the *P. pastoris* yeast expression host. Treatment of 10μg of recombinant avidin with 500 units of PNGase F overnight at 37°C to remove N-glycan chains resulted in a reduction of the intensity of the 20kDa recombinant avidin band seen on sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) gels and the appearance of a band at 16kDa (Figure 3.1). The presence of some remaining 20kDa recombinant avidin indicates that the reaction had not run to completion. Yeast invertase is known to be highly glycosylated; hence it was included as a positive control. The appearance of one band at 64kDa indicates the yeast invertase was completely converted to a deglycosylated form.

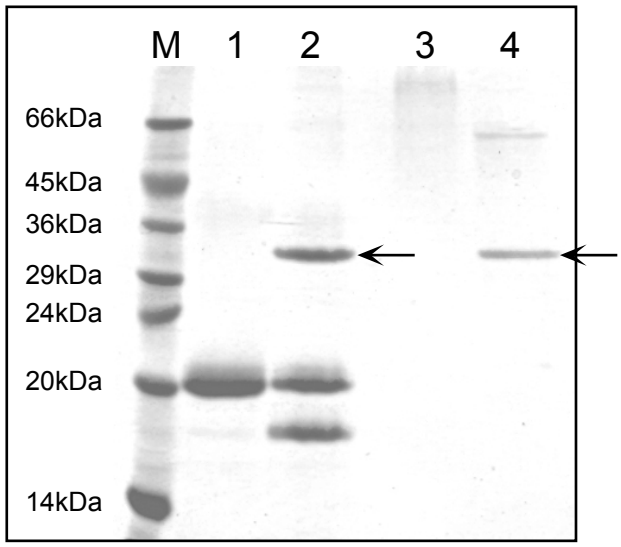


Figure 3.1.

15% SDS-PAGE analysis following *in vitro* deglycosylation of recombinant avidin by PNGase F.

M is SDS7 molecular weight marker. Lane 1 is recombinant avidin (20kDa). Lane 2 is deglycosylated recombinant avidin (16kDa). The presence of a band at 20kDa and 16kDa indicates the reaction had not run to completion. Lane 3 is yeast invertase (135kDa). Lane 4 is deglycosylated yeast invertase (64kDa). (20μl loaded for each sample). The arrows indicate the PNGase F enzyme.

2. Gel Filtration

Gel filtration of 2mg of recombinant avidin and commercial egg-white avidin was carried out through a sephacryl S-200 column using phosphate buffered saline (PBS). The two proteins gave identical elution profiles, with both samples running at a molecular weight of approximately 60kDa when compared to a standard protein calibration (Figure 3.2). This suggests that recombinant avidin forms tetrameric molecules similar to egg-white avidin.

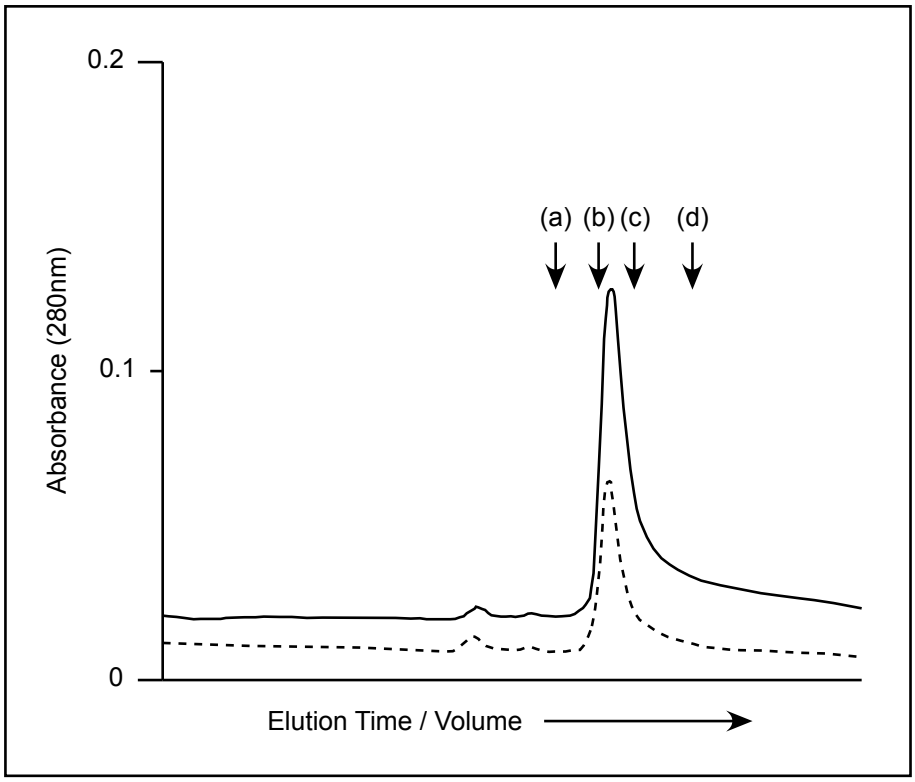


Figure 3.2.

Sephacryl S-200 gel filtration profile of avidin.

The dashed line represents commercial egg-white avidin. The solid line represents recombinant avidin. The labelled arrow-heads indicate the elution points of standard proteins used for calibration: (a) bovine serum albumin dimer (134kDa), (b) bovine serum albumin (67kDa), (c) ovalbumin (45kDa), (d) soya bean Kunitz trypsin inhibitor (20kDa).

3. Binding to Biotin

The ability of recombinant avidin to bind biotin was confirmed *in vitro* by incubation with a molar excess of immobilised biotin (agarose-biotin). The immobilised biotin matrix was mixed with 5μg of recombinant avidin for 30 minutes at room temperature to ensure complete binding. Following microcentrifugation, the matrix pellet was washed twice with PBS. Finally, any bound recombinant avidin was eluted from the matrix by boiling. Analysis by SDS-PAGE (Figure 3.3) showed the absence of recombinant avidin in the unbound fraction, an absence of protein in the wash fractions and 100% recombinant avidin eluted upon boiling. This shows that all of the recombinant avidin was bound to the biotin matrix and suggests that it was fully functional.

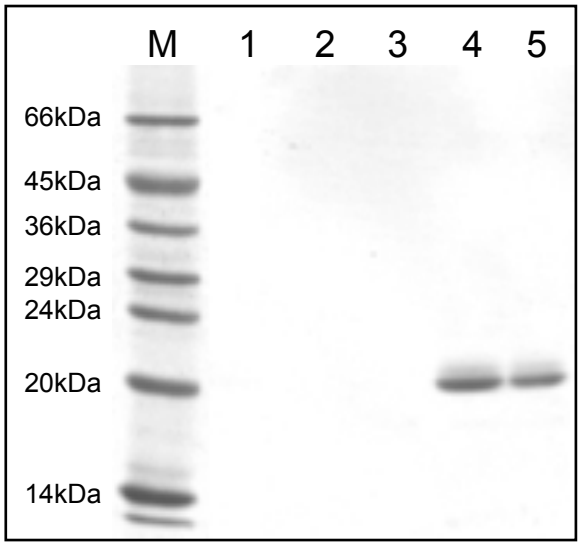


Figure 3.3.

15% SDS-PAGE analysis following *in vitro* binding of recombinant avidin to immobilised biotin (agarose-biotin).

M is SDS 7 molecular weight marker. Lane 1 is the unbound fraction. Lanes 2 and 3 are the PBS wash fractions. Lane 4 is the elution fraction, showing recombinant avidin (20kDa). (25μl loaded for each sample). Lane 5 is 5μg of recombinant avidin (20kDa).

Insecticidal Activity of Orally Delivered Recombinant Avidin Towards Two Hemipteran Species

The oral toxicity of avidin towards two hemipteran pest species was evaluated by allowing *Acyrthosiphon pisum* (pea aphid) (Hemiptera: Aphididae, Harris 1776) and *Sitobion avenae* (cereal aphid) (Hemiptera: Aphididae, Fabricius 1775) to feed on artificial diets containing recombinant avidin at concentrations in the range 0.05mg/ml-2mg/ml (50ppm-2000ppm, 0.75μM-30μM). Artificial diet was used as a negative control for the feeding bioassay. The standard aphid diet contained a background level of biotin of 0.001mg/ml (4μM).

Recombinant avidin showed a dose-dependent insecticidal activity towards *A. pisum*, causing a reduction in aphid survival (Figure 3.4). Acute toxicity was observed on diets containing 1mg/ml, 0.5mg/ml and 0.25mg/ml recombinant avidin, with 100% mortality recorded for all three treatments in four days (Mantel-Cox log-rank statistical analysis, compared to artificial diet control, $P<0.0001$). Aphid survival was also reduced in treatments containing 0.2mg/ml, 0.1mg/ml and 0.05mg/ml recombinant avidin, with respective values of 20%, 70% and 80% recorded at the end of the 10-day bioassay, compared to 95% control aphid survival. Differences in survival were statistically significant for the 0.2mg/ml and 0.1mg/ml treatments (Mantel-Cox log-rank statistical analysis, $P<0.0001$ and $P<0.0087$ respectively), but not for the 0.05mg/ml treatment (Mantel-Cox log-rank statistical analysis, $P=0.1527$) when compared to controls. For these lower doses, aphid mortality was observed in the first six days of the bioassay, after which, no further decrease in survival was recorded. Fitting data from the mid-point of the bioassay (day 5) to a dose-response curve equation allowed a median lethal concentration (LC_{50}) of 0.14mg/ml (140ppm, 2.1μM) for recombinant avidin toxicity to pea aphid to be estimated (Figure 3.5). This LC_{50} was also similar for data from the end of the bioassay (day 10).

Supplementation of the aphid diet with biotin alleviated the insecticidal effects of recombinant avidin. Pea aphids fed diets containing recombinant avidin and biotin at concentrations of 2mg/ml and 0.1mg/ml respectively (2000ppm and 100ppm, 30μM and 410μM respectively), showed no reduction in survival when

compared to controls. This suggests that the insecticidal activity of recombinant avidin towards *A. pisum* was attributable to a biotin deficiency caused by the sequestering of biotin by avidin.

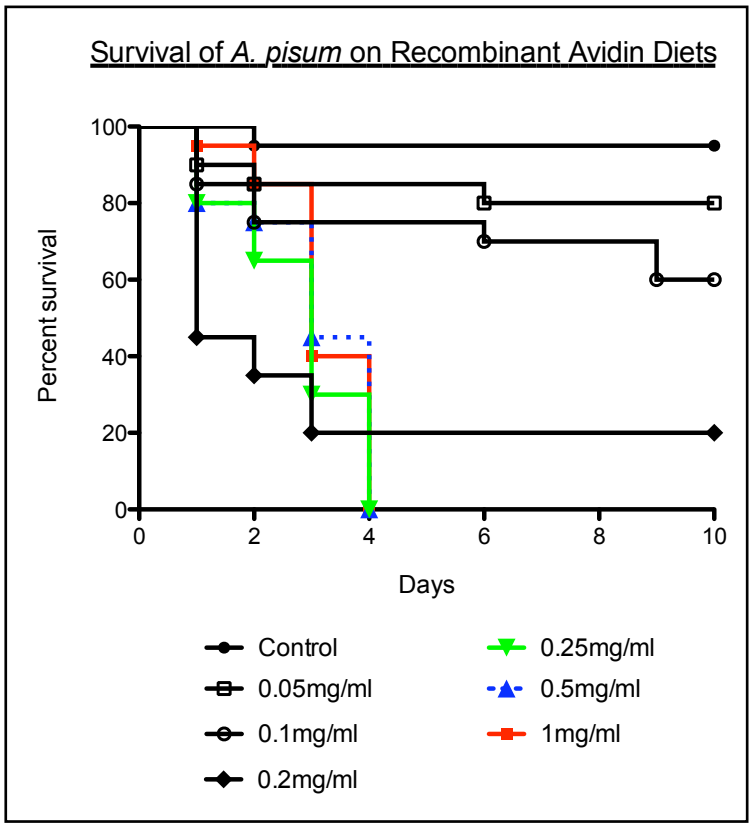


Figure 3.4.

Survival of *A. pisum* (pea aphid) fed on artificial diet containing varying concentrations of recombinant avidin.
(n = 20 for each concentration - Two separate treatments with 10 individuals).

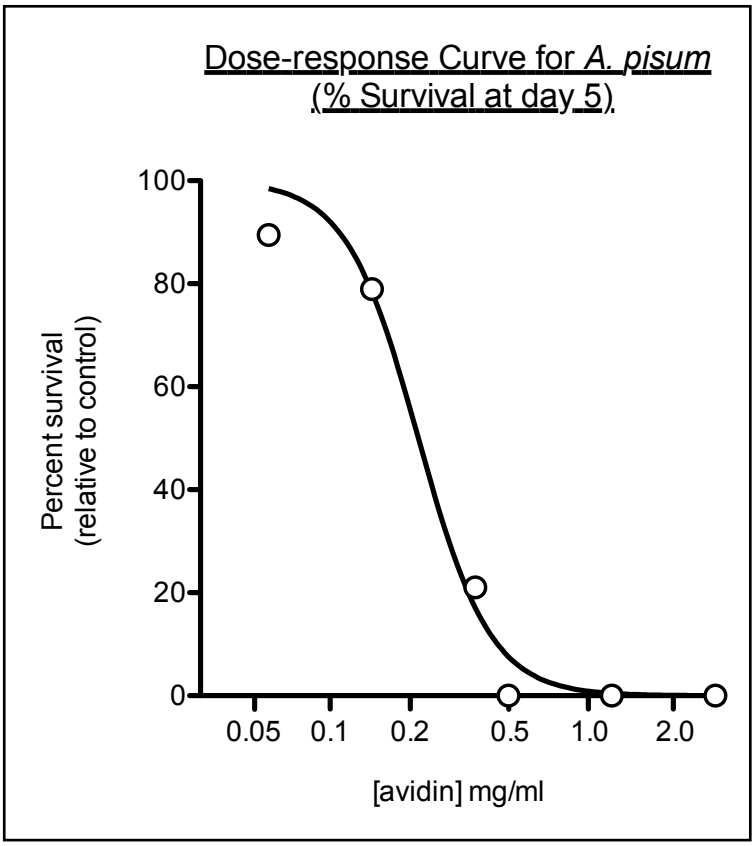


Figure 3.5.

Dose response curve for *A. pisum* at day 5 of recombinant avidin feeding assay.
The data are fitted to a standard dose-response curve using non-linear regression.
(n = 20 for each concentration - Two separate treatments with 10 individuals).

In contrast to the effects on *A. pisum*, when recombinant avidin was fed to *S. avenae*, no significant differences on aphid survival were observed at concentrations up to 1mg/ml (1000ppm, 15μM). Even when recombinant avidin was incorporated into diet at a concentration of 2mg/ml (2000ppm, 30μM), aphid survival after 10 days of feeding was only reduced to 75%, compared to 95% for control insects (Figure 3.6). However, avidin did have an effect on the growth of *S. avenae*, since all of the aphids exposed to diets containing recombinant avidin at 2mg/ml (2000ppm, 30μM) were observed to be around 40% of the size of the control aphids at the end of the 10-day bioassay (Figure 3.7).

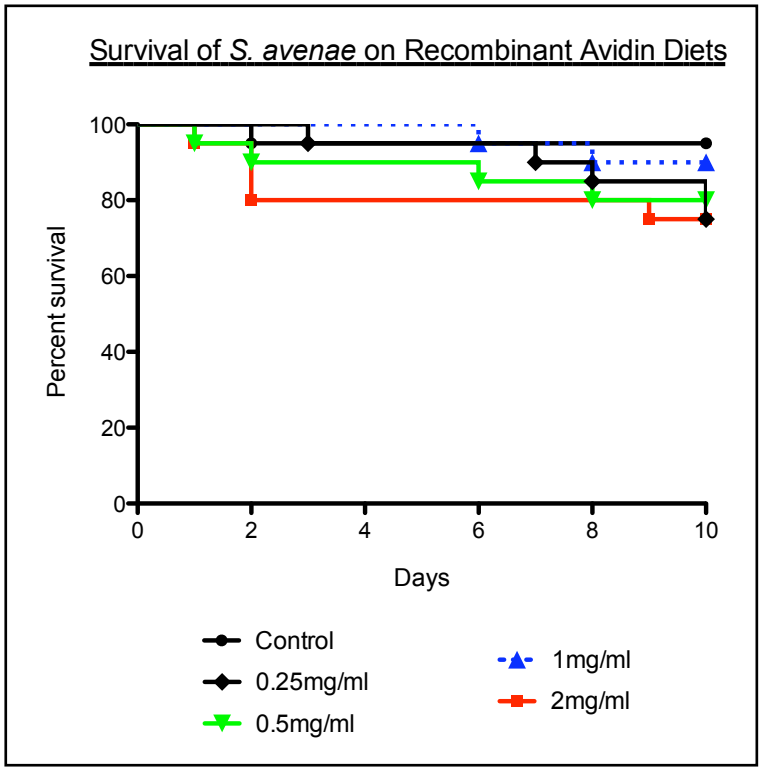
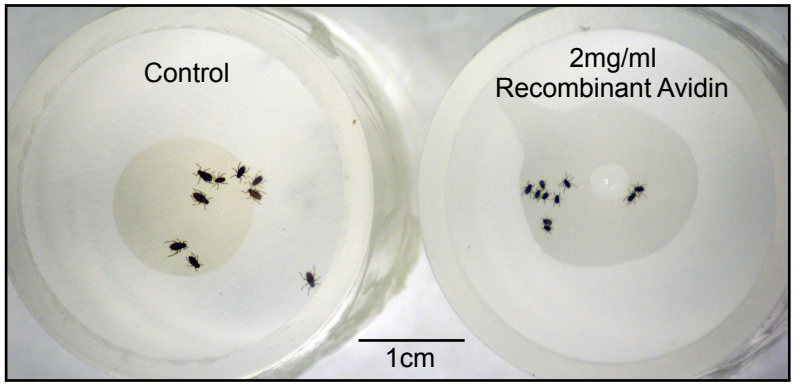


Figure 3.6.

Survival of *S. avenae* (cereal aphid) fed on artificial diet containing varying concentrations of recombinant avidin.
(n = 20 for each concentration - Two separate treatments with 10 individuals).

(a)



(b)

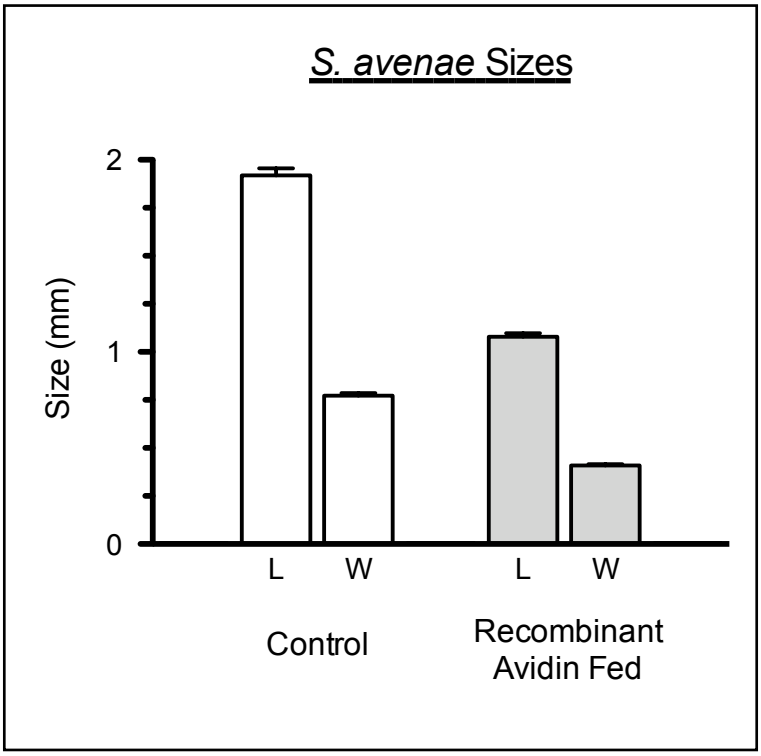


Figure 3.7.

Size difference of *S. avenae* fed on control diet or on artificial diet containing 2mg/ml recombinant avidin.

(a) Photographic comparison of *S. avenae* in aphid feeding chambers. (b) Graphic representation of the average size of the aphids measured using ImageJ (L = Length, W = Width) (n = 20 for each treatment - Two separate treatments with 10 individuals).

Mechanism of Toxicity of Recombinant Avidin Towards *A. pisum*

1. Difference in Stability of Recombinant Avidin in the Gut of *A. pisum* and *S. avenae*

Whole insect protein extracts prepared from aphids exposed to dietary recombinant avidin showed no differences in proteolytic degradation between *A. pisum* and *S. avenae*. Protein extracts produced directly from aphids fed diets containing recombinant avidin at 0.5mg/ml (500ppm, 7.5μM) for 48 hours and also from aphids that had been chase-fed with control diet for 48 hours post recombinant avidin feeding, were analysed by western blotting (anti-avidin antibodies) (Figure 3.8).

The blot suggests that the contrast in insecticidal activity is not attributable to differences in the susceptibility of the protein to gut proteolytic degradation *in vivo* in the different aphid species, as the same bands on the blot are present in both. However, in contrast to the recombinant avidin added to the diet (20kDa), the avidin in both aphids extracts shows signs of proteolytic degradation (19kDa, 15kDa and to a lesser extent, 10kDa), with all three products still being immunoreactive with the avidin antibody. Although the levels of degraded recombinant avidin detected in extracts from aphids that had been chase-fed with control diet were lower than in aphids without the chase, the protein was still present in readily detectable amounts, suggesting that ingested avidin had bound to the gut or had been sequestered, and that not all of the ingested protein had been excreted in honeydew within 24 hours. No avidin was detected in aphids fed artificial diet as a control. The blot also shows the presence of an immunoreactive, higher molecular weight protein (36kDa), suggesting the formation of a recombinant avidin dimer as it is not present in protein extracts obtained from aphids fed with control diet (Figure 3.8).

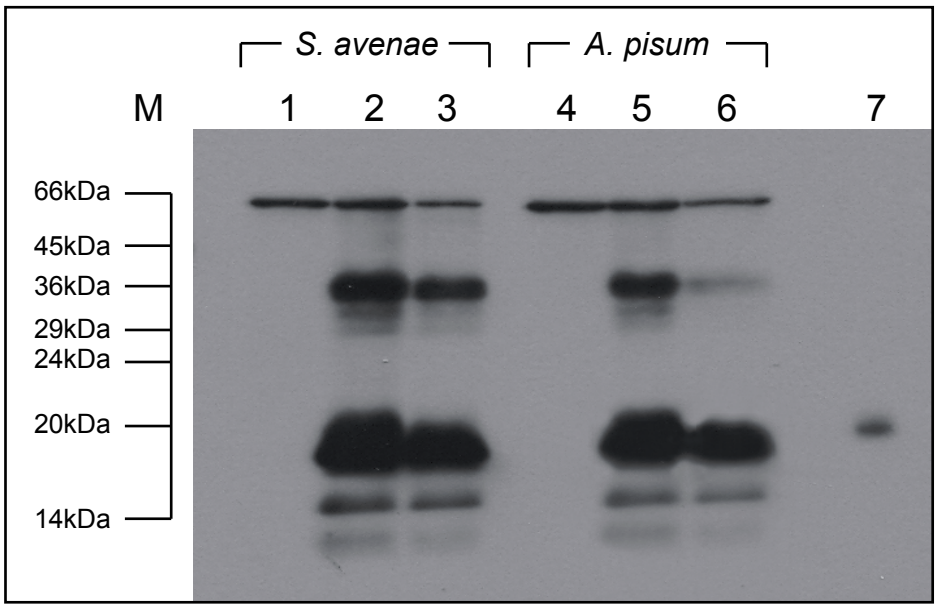


Figure 3.8.

Western analysis of whole *S. avenae* and *A. pisum* protein extracts prepared directly after feeding on 0.5mg/ml recombinant avidin for 48 hours, or after chasing recombinant avidin with control diet for 48 hours.

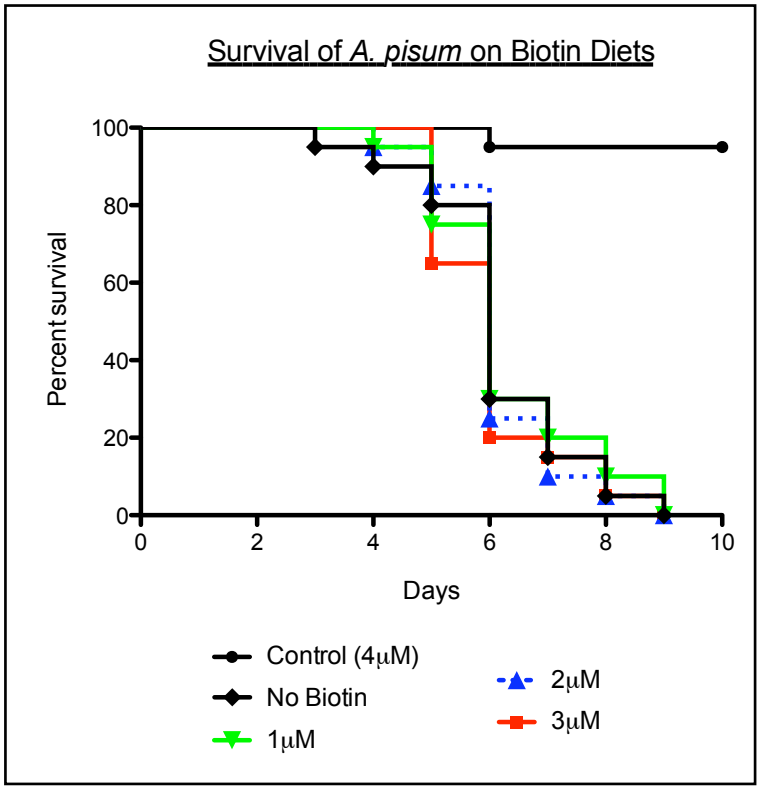
A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is protein extract from *S. avenae* fed with control diet for 48 hours. Lane 2 is protein extract from *S. avenae* fed with recombinant avidin for 48 hours. Lane 3 is protein extract from *S. avenae* fed with recombinant avidin for 48 hours followed by control diet for 48 hours. The avidin in the aphid extracts shows signs of proteolytic degradation (19kDa, 15kDa and 10kDa). There is also a higher molecular weight protein (36kDa), suggesting the formation of a recombinant avidin dimer. Lanes 4-6 are the equivalent protein extracts from *A. pisum*. (25µl loaded for each sample). Lane 7 is 10ng of recombinant avidin (20kDa).

2. Difference in Susceptibility to Biotin Depletion in *A. pisum* and *S. avenae*

As avidin is known to sequester biotin, an investigation into the effect of dietary biotin was conducted. When *A. pisum* and *S. avenae* were fed on diets containing varying amounts of biotin, from 0µM up to 4µM biotin (4ppm, 0.004mg/ml) (normal artificial diet concentration), mortality was observed in both species at biotin levels of 3µM or less (Figure 3.9). Pea aphids fed diet containing the normal 4µM amount of biotin grew normally with no mortality, whereas aphids fed levels of 3µM or less of biotin showed similar mortality to aphids fed no biotin at all, with 100% mortality observed by day nine for all three treatments. Cereal aphids were less sensitive to reduced biotin levels in diet, with 100% mortality at day nine

and similar survival curves for treatments in the range 0μM-2μM, but only partial mortality (55%) for the 3μM biotin treatment (Mantel-Cox log-rank statistical analysis, P=0.0001, significant difference between both the 3μM treatment and the control and, P=0.0291, for the 3μM and 0μM-2μM biotin treatments). Survival and growth of the aphids fed the normal 4μM diet was similar to that for *A. pisum*.

(a)



(b)

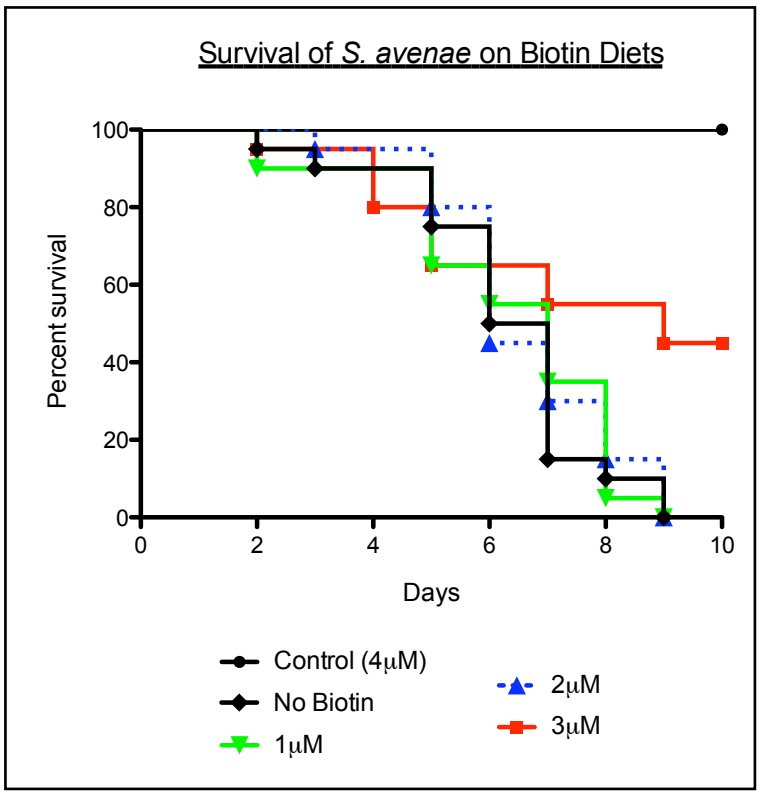


Figure 3.9.

Survival of aphids fed on artificial diet containing varying concentrations of biotin. (a) *A. pisum*. (b) *S. avenae.* (n = 20 for each concentration - Two separate treatments with 10 individuals).

The two aphid species also showed differing responses when fed diet containing no biotin and a sub-lethal level of recombinant avidin (0.2mg/ml for *A. pisum* and 2mg/ml for *S. avenae*) (Figure 3.10). In this assay, 100% mortality was observed for both aphid species in 9-10 days for the diets with an absence of biotin. Adding 0.2mg/ml recombinant avidin to the biotin-free diet accelerated the complete mortality of *A. pisum* to seven days. In contrast, the addition of 2mg/ml recombinant avidin to the *S. avenae* biotin-free diet promoted a statistically significant 75% survival outcome at day 10 (Mantel-Cox log-rank statistical analysis, compared to no biotin control, $P<0.0001$). This suggests that supplying the recombinant avidin to *S. avenae* compensates largely for the absence of biotin.

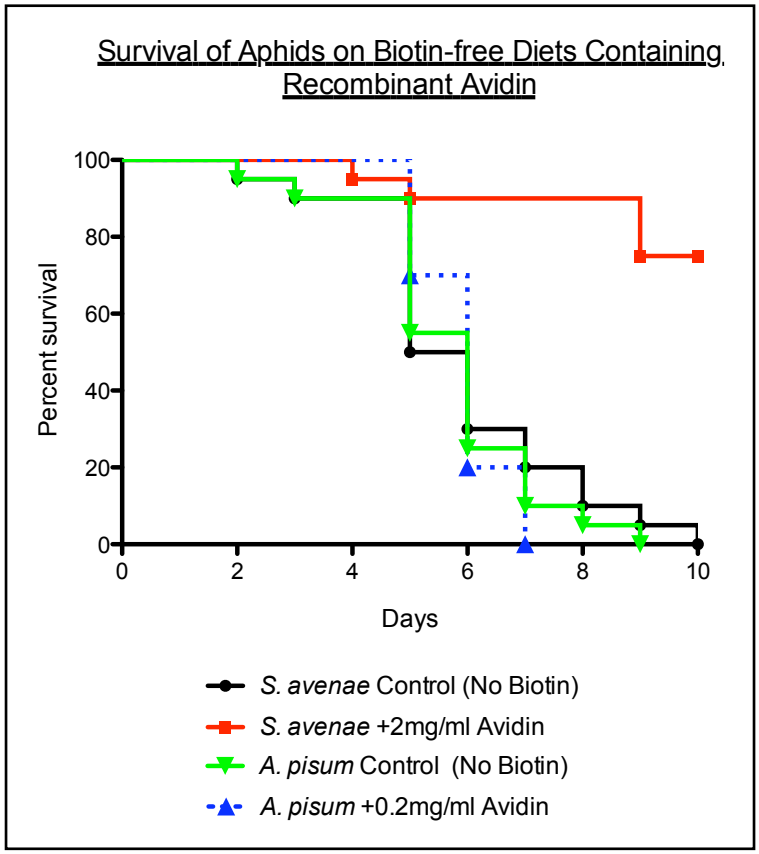


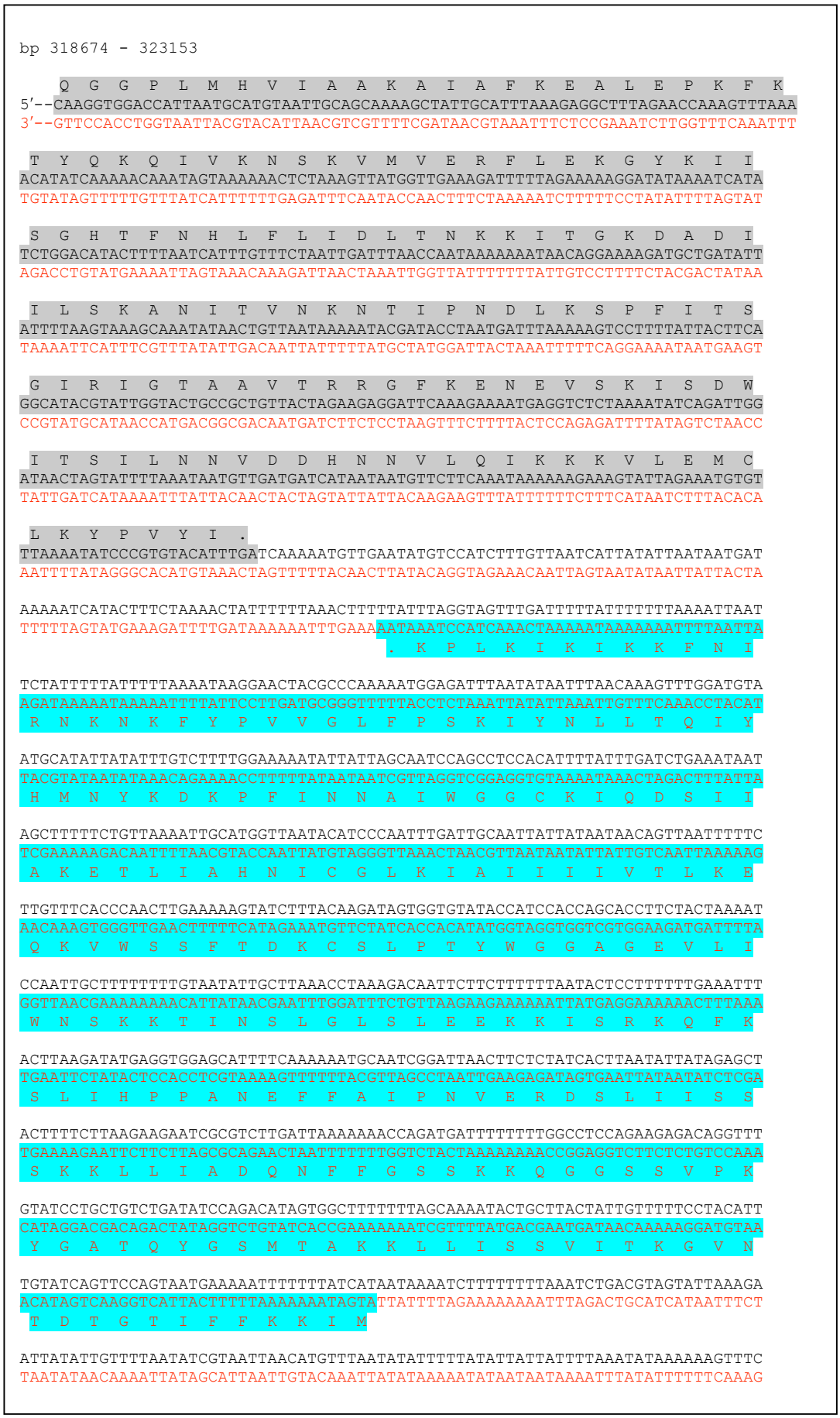
Figure 3.10.

Survival of aphids fed on biotin-free artificial diet containing recombinant avidin.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

3. Difference in Biotin Synthesis by Bacterial Symbionts in *A. pisum* and *S. avenae*

To investigate one other possibility that *S. avenae* survived better on reduced biotin diets because the *Buchnera* symbionts contain a functional operon for biotin synthesis, the sequence of the region of *Buchnera* genome containing the biotin operon was determined. A fragment of approximately 4.5kbp was amplified from DNA isolated from *S. avenae*, using primers based on the published *A. pisum Buchnera* genome sequence (Chapter 2). This fragment was fully sequenced (Figure 3.11).

The determined sequence was very similar to the published genome sequence for *Buchnera aphidicola* from *A. pisum*, containing parts of the *glyA* and *ybhE* genes which flank the *bio* operon and open reading frames for the polypeptides encoded by the *bioA*, *bioB* and *bioD* genes (Figure 3.11). This result shows that the *S. avenae* symbionts, like the *A. pisum* symbionts, lack the full complement of genes necessary for biotin synthesis (*bioW* and *bioF* missing).


bp 318674 - 323153
Q G G P L M H V I A A K A I A F K E A L E P K F K
5′--CAAGGTGGACCATTAATGCATGTAATTGCAGCAAAAGCTATTGCATTTAAAGAGGCTTTAGAACCAAAGTTTAAA
3′--GTTCCACCTGGTAATTACGTACATTAACGTCGTTTTCGATAACGTAAATTTCTCCGAAATCTTGGTTTCAAATTT
T Y Q K Q I V K N S K V M V E R F L E K G Y K I I
ACATATCAAAAACAAATAGTAAAAAACTCTAAAGTTATGGTTGAAAGATTTTTAGAAAAAGGATATAAAATCATA
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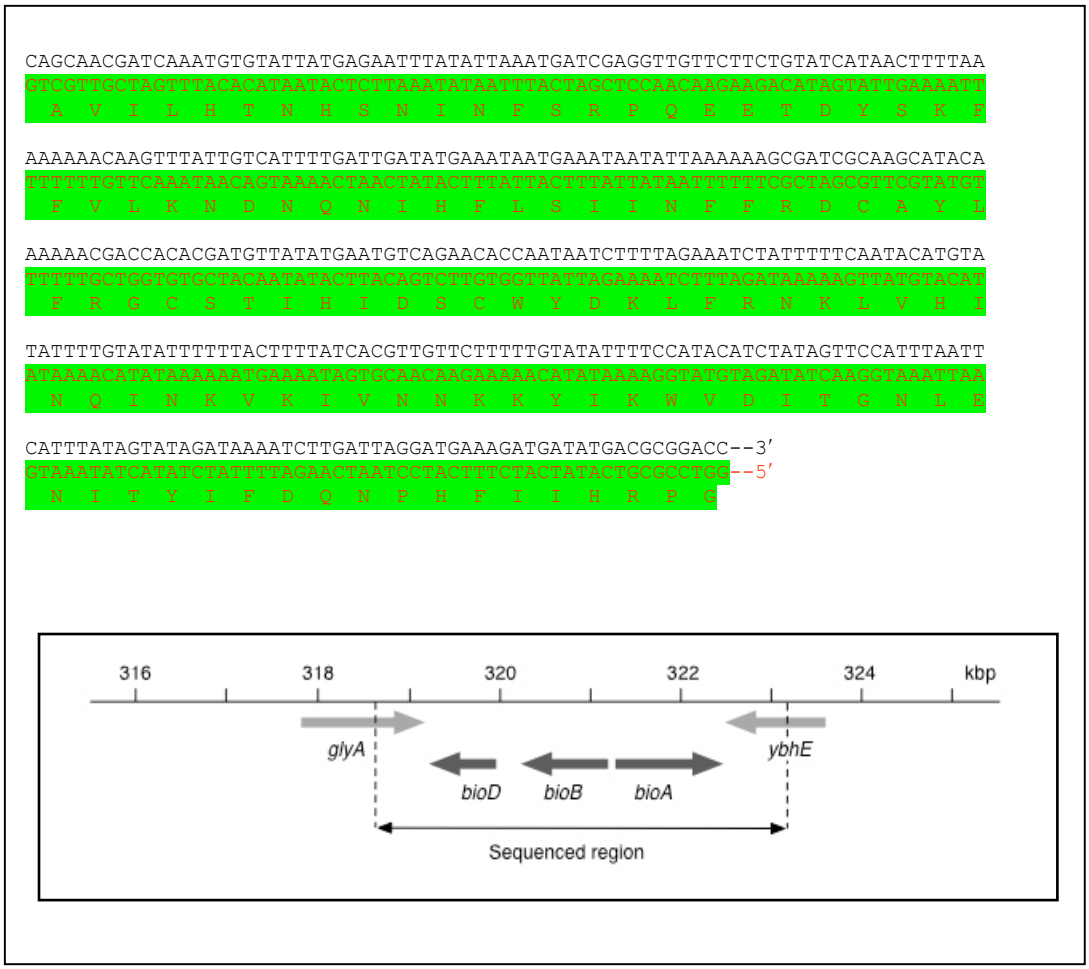


Figure 3.11.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the region of genome sequenced in *Buchnera* from *S. avenae*.

(From *Buchnera aphidicola*; accession CP001158).

(5′-3′) The *glyA* gene flanking the *bio* operon is highlighted in grey (bp 318674-319147 truncated). The *bioA* gene is highlighted in purple (bp 321382-322668).

(3′-5′) The *ybhE* gene flanking the *bio* operon is highlighted in green (bp 323153-322677 truncated). The *bioB* gene is highlighted in yellow (bp 321303-320272). The *bioD* gene is highlighted in blue (bp 319909-319235). Diagram not to scale.

Transport of Recombinant Avidin in Insects

1. Hemipteran *In vivo* Transport of Recombinant Avidin

Analysis of guts and haemolymph extracted from *A. pisum* following ingestion of recombinant avidin revealed it was likely that recombinant avidin was not transported throughout the aphid body and haemolymph *in vivo*. Extractions were carried out on aphids fed on diet containing 1mg/ml (1000ppm, 15μM) recombinant avidin for 24 and 48 hours and also from aphids that had been chase-fed with control diet for 24 hours post recombinant avidin feeding.

Avidin was readily detected in the dissected guts after a chase of 24 hours (Figure 3.12). On the other hand however, no avidin could be detected in the haemolymph extracted from the aphids. These results suggest that recombinant avidin is being retained in the gut, but is not transported to the haemolymph. As observed previously, in contrast to the recombinant avidin added to the diet (20kDa), the avidin in the aphid gut shows signs of proteolytic degradation (19kDa) and dimerisation (36kDa) (Figure 3.12).

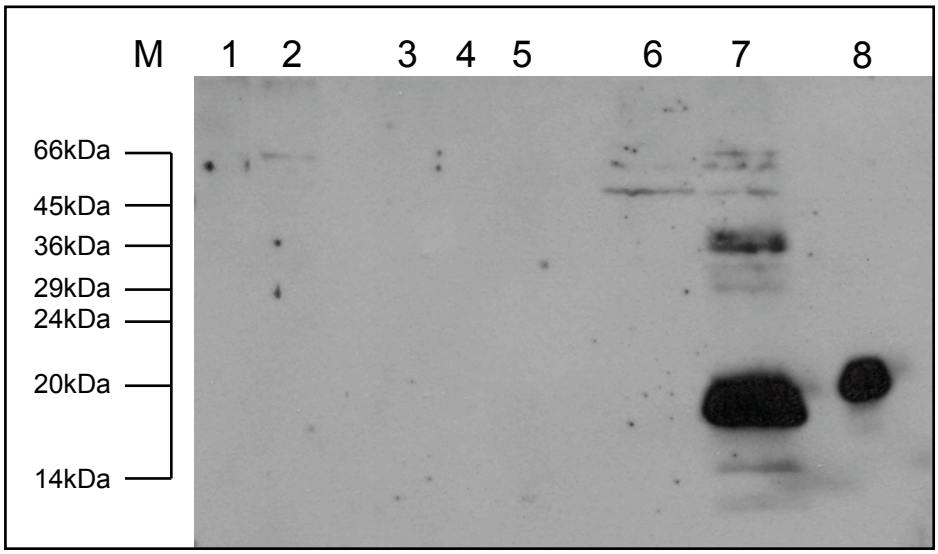


Figure 3.12.

Western analysis of *A. pisum* haemolymph and extracted guts after feeding on 1mg/ml recombinant avidin for 24 hours, 48 hours or after chasing recombinant avidin with control diet for 24 hours.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is haemolymph from aphids fed with control diet for 24 hours. Lane 2 is haemolymph from aphids fed with recombinant avidin for 24 hours. Lane 3 is haemolymph from aphids fed with control diet for 48 hours. Lane 4 is haemolymph from aphids fed with recombinant avidin for 48 hours. Lane 5 is haemolymph from aphids fed with recombinant avidin for 48 hours followed by control diet for 24 hours. Lane 6 is guts from aphids fed with control diet for 48 hours. Lane 7 is guts from aphids fed with recombinant avidin for 48 hours followed by control diet for 24 hours. The avidin in the aphid gut shows signs of proteolytic degradation (19kDa) and dimerisation (36kDa). (25µl loaded for each sample). Lane 8 is 10ng of recombinant avidin (20kDa).

To identify the site of avidin binding, *A. pisum* were fed diet containing fluorescently-labelled recombinant avidin (fluorescein isothiocyanate-recombinant avidin; FITC-recombinant avidin). Fluorescein isothiocyanate (FITC) was used as a control. The labelled proteins were included in artificial diet at a concentration of 0.2mg/ml (200ppm) and fed for 48 hours. Half of the aphids were also chase-fed with control diet for 24 hours. Following the feeding treatments, whole aphids or dissected guts were observed directly under the fluorescence microscope and images were captured in Openlab (Chapter 2) (Figure 3.13).

After feeding, FITC was observed throughout the gut, but all of the fluorescence was eliminated from the aphid after 24 hours chase with label-free diet,

showing that the label itself was not subject to non-specific binding. The FITC-recombinant avidin was strongly bound by the anterior region (foregut) of the aphid gut when fed and could be detected readily *in vivo* even after 24 hours chase with diet containing no labelled components (Figure 3.13).

As shown in Figure 3.14, when guts were dissected from aphids fed FITC-recombinant avidin, all of the fluorescence observed was present in the stomach region (foregut) of the aphid gut and no fluorescence was observed in the midgut region. The fluorescence appeared to be associated with the gut cell surface, although attempts to visualise this directly were unsuccessful. These results strongly suggest that avidin is being retained in the aphid stomach. The retention of avidin in the aphid was also observed when the recombinant avidin was saturated with biotin pre-feeding, showing that the binding to the gut is not dependent on avidin's biotin-binding capacity.

Similar results were also observed in *S. avenae*, suggesting that the avidin binding to the stomach may be common throughout the hemipteran order.

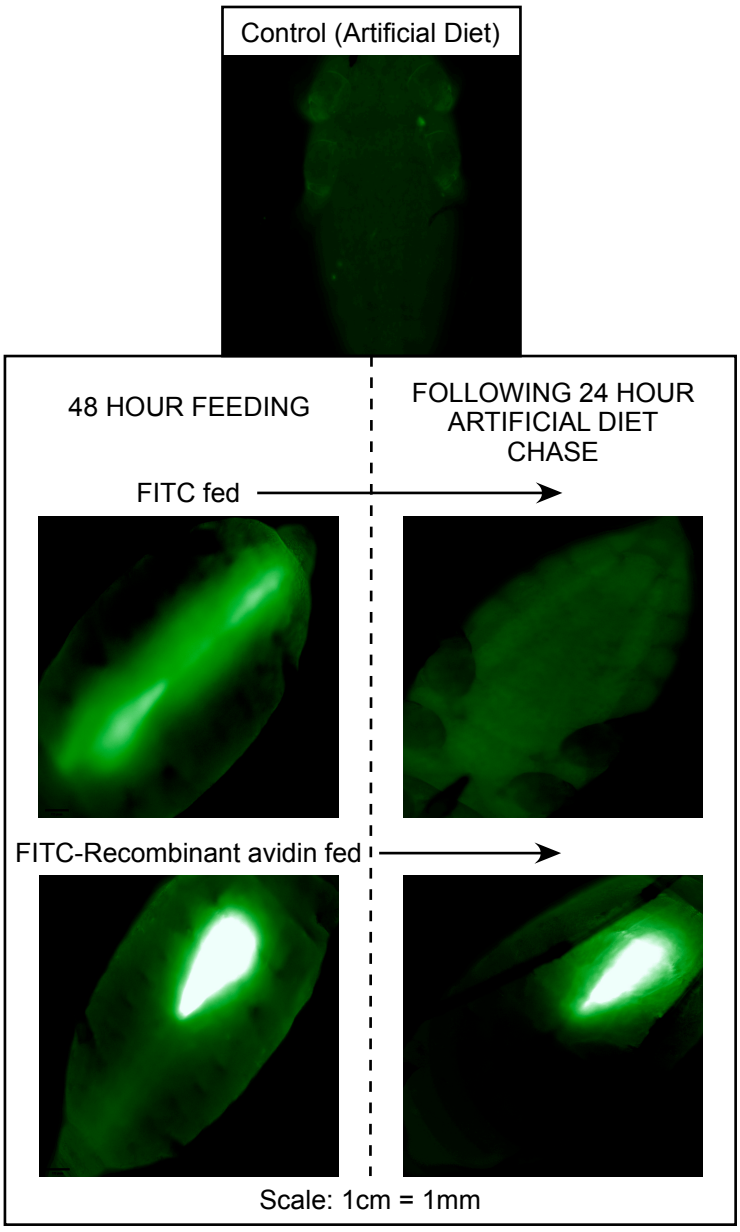


Figure 3.13.

Fluorescence micrographs of *A. pisum* after feeding on 0.2mg/ml FITC or FITC-labelled recombinant avidin.

(Left hand side) After feeding for 48 hours. (Right hand side) After chasing fluorescent label with control diet for 24 hours.

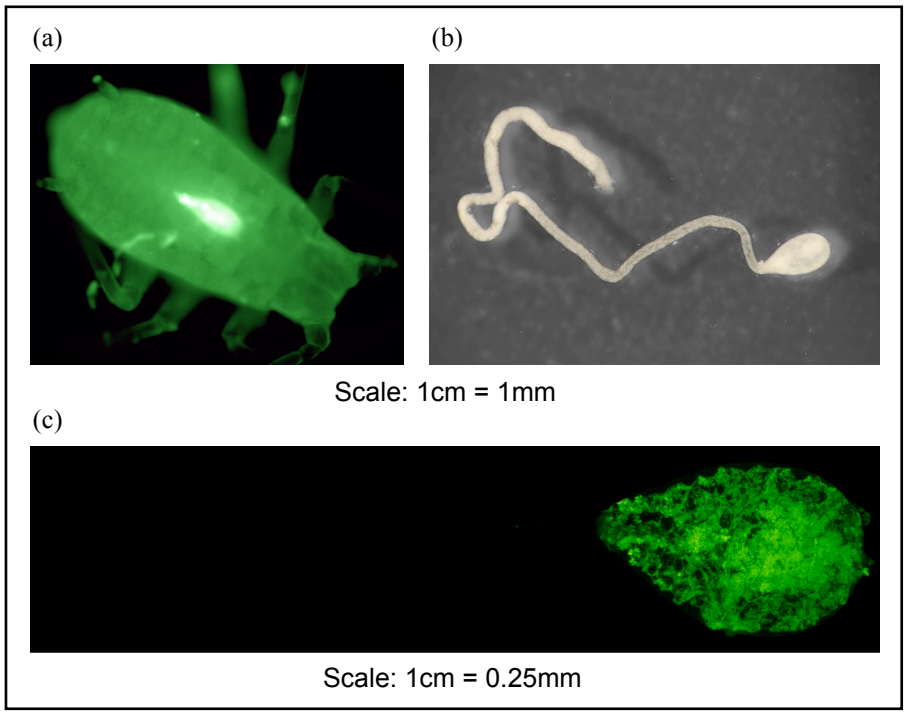


Figure 3.14.

Micrographs showing binding of FITC-labelled recombinant avidin to aphid gut.
(a) Fluorescence micrograph of a whole aphid after feeding on FITC-labelled recombinant avidin for 48 hours followed by control diet for 24 hours. (b) Micrograph of a dissected aphid gut. (c) Fluorescence micrograph of a dissected aphid gut after feeding on FITC-labelled recombinant avidin for 48 hours followed by control diet for 24 hours.

Further analysis of *A. pisum* gut protein extracts from a recombinant avidin feeding timecourse experiment with an extended chase period showed that recombinant avidin is retained in the gut for at least 72 hours. As with the initial transport experiment, 1mg/ml (1000ppm, 15μM) recombinant avidin was fed to aphids for 24 hours and guts were extracted following a chase with control diet for either 24, 48 or 72 hours.

As observed previously, in contrast to the recombinant avidin added to the diet (20kDa), the avidin in the aphid gut shows signs of proteolytic degradation (19kDa, 15kDa and 10kDa) and dimerisation (36kDa) (Figure 3.15). However, the avidin was still observed after 72 hours, with only a limited decrease in the amount, confirming the binding in the gut and supporting the likelihood of it remaining present post 72 hours (Figure 3.15).

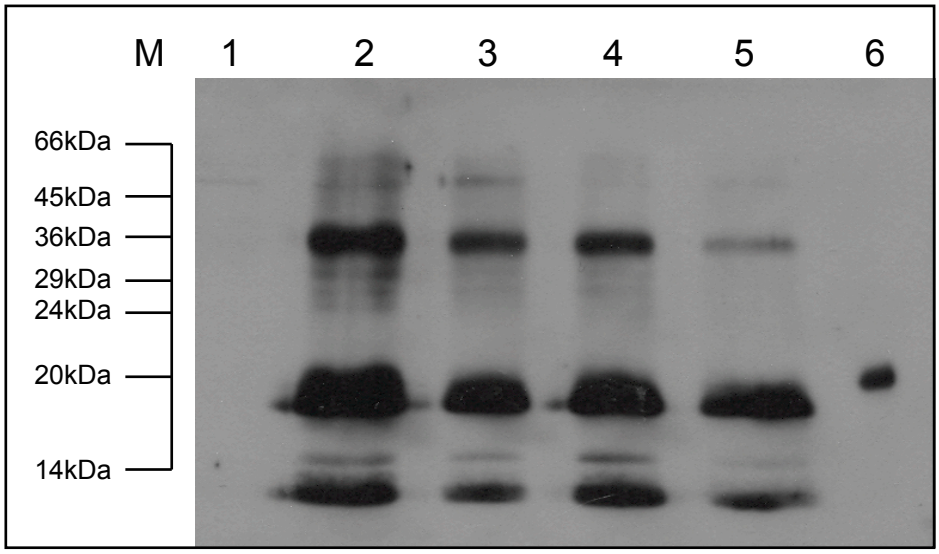


Figure 3.15.

Western analysis of *A. pisum* extracted guts after feeding on 1mg/ml recombinant avidin for 24 hours and chasing recombinant avidin with control diet for 24 hours, 48 hours and 72 hours.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is guts from aphids fed with control diet for 72 hours. Lane 2 is guts from aphids fed with recombinant avidin for 24 hours. Lane 3 is guts from aphids fed with recombinant avidin for 24 hours followed by control diet for 24 hours. Lane 4 is guts from aphids fed with recombinant avidin for 24 hours followed by control diet for 48 hours. Lane 5 is guts from aphids fed with recombinant avidin for 24 hours followed by control diet for 72 hours. (25μl loaded for each sample). The avidin in all of the samples shows signs of proteolytic degradation (19kDa, 15kDa and 10kDa). There is also a higher molecular weight protein (36kDa), suggesting the formation of a recombinant avidin dimer. Lane 6 is 10ng of recombinant avidin (20kDa).

To identify the type of avidin interaction within the aphid gut, a pull-down assay of *A. pisum* gut tissue was carried out. Approximately 100 adult *A. pisum* guts were dissected and homogenised in PBS containing Triton X-100. Supernatant containing the extracted gut proteins was incubated with recombinant avidin labelled magnetic beads overnight at 4°C. Following incubation, the beads were washed three times with PBS and analysed by silver stained SDS-PAGE (Figure 3.16).

After incubation with recombinant avidin labelled magnetic beads, it is clear that extracted *A. pisum* gut proteins do not interact with avidin *in vitro*. The complex mix of proteins in the gut tissue preparation can be seen in the unbound and wash

fractions whilst the elution fraction is identical to the starting magnetic beads (Figure 3.16).

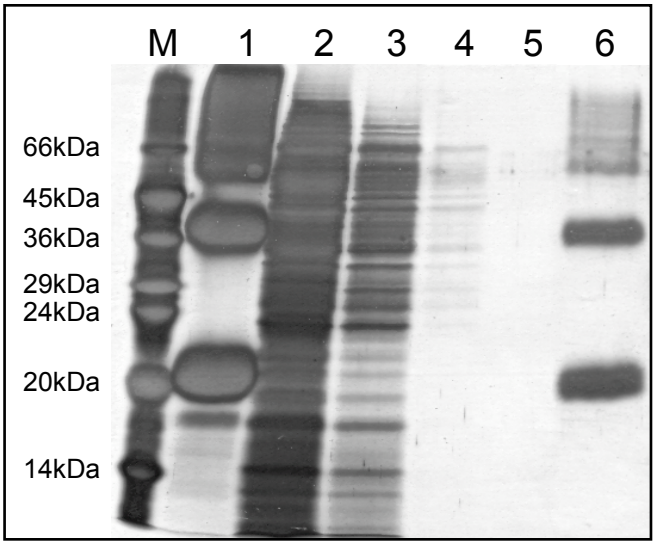


Figure 3.16.

15% SDS-PAGE analysis following an *A. pisum* gut tissue pull-down assay with recombinant avidin labelled magnetic beads.

(silver stained).

M is SDS 7 molecular weight marker. Lane 1 is recombinant avidin labelled magnetic beads. Recombinant avidin is 20kDa. There is also a higher molecular weight protein (36kDa), suggesting the formation of a recombinant avidin dimer during the labelling. Lane 2 is applied extracted gut proteins. Lane 3 is the unbound fraction. Lanes 4 and 5 are the PBS wash fractions. Lane 6 is the elution fraction, showing both recombinant avidin (20kDa) and the recombinant avidin dimer (36kDa). (25µl loaded for each sample).

Far western blotting was used to confirm that avidin would not bind to gut proteins *in vitro*. Extracted gut proteins from approximately 40 *A. pisum* were transferred to nitrocellulose membrane prior to blocking of the non-specific binding sites with bovine serum albumin (BSA). The membrane was then incubated with recombinant avidin and following extensive washing, was probed with anti-avidin antibodies to detect any bound avidin. As a positive control, a similar blot was also conducted with *Galanthus nivalis* agglutinin (GNA), which is known to bind to mannose residues within the aphid gut.

The result (Figure 3.17) further supports the gut pull-down assay finding that extracted gut proteins do not bind to avidin *in vitro*. No extra bands are visible when

compared to the negative control. However, the GNA positive control clearly shows a different banding pattern, indicating the occurrence of protein interactions.

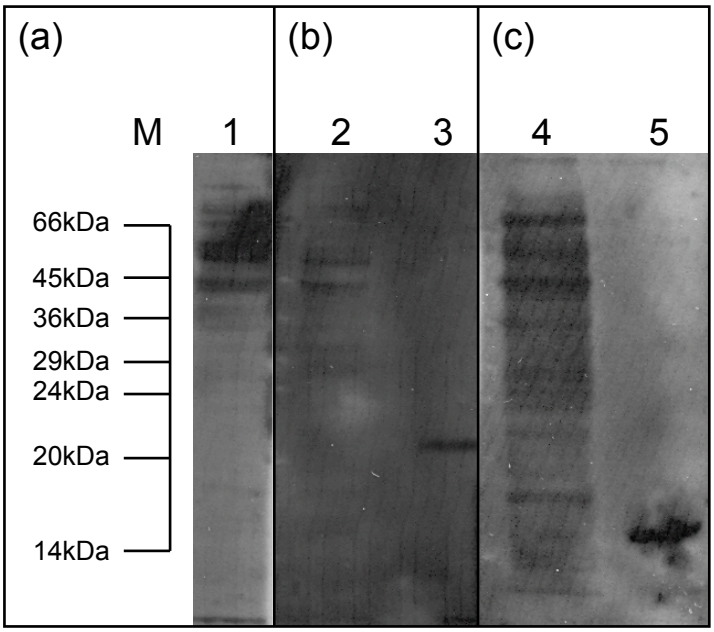


Figure 3.17.

Far western analysis of *A. pisum* extracted guts.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel is shown. M is SDS 7 molecular weight marker. (a) Control (no probe). Lane 1 is control guts. (b) Probed with recombinant avidin (1:2000) followed by anti-avidin antibodies (1:10000). Lane 2 is avidin probed guts. Lane 3 is 10ng of recombinant avidin (20kDa). (c) Probed with recombinant GNA (1:2000) followed by anti-GNA antibodies (1:3000). Lane 4 is GNA probed guts. (25µl loaded for each gut sample). Lane 5 is 50ng of recombinant GNA (15kDa).

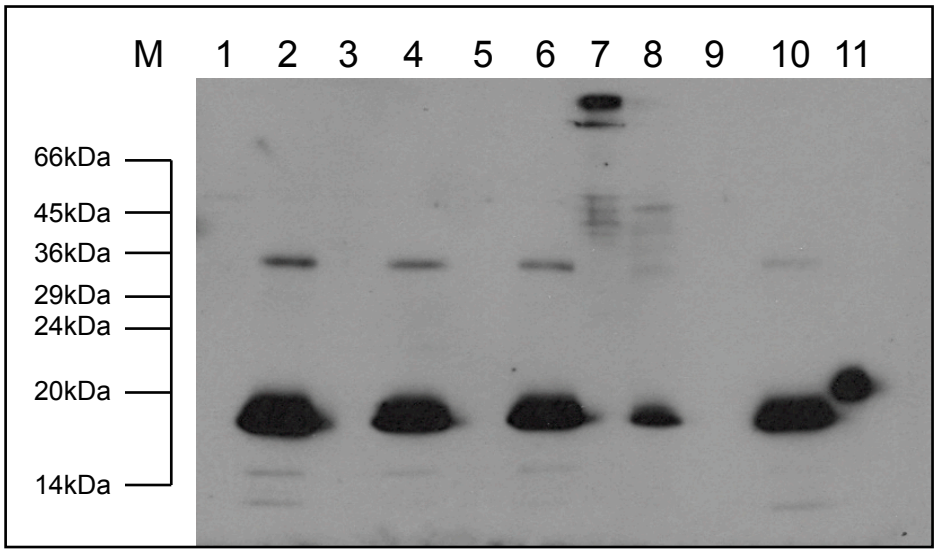
2. Lepidopteran *In vivo* Transport of Recombinant Avidin

The analysis of dissected lepidopteran larvae components following feeding on diet containing recombinant avidin suggests that avidin is readily transported throughout the whole insect. Dissections were carried out on fifth stadium *M. brassicae* larvae fed on diet containing 1000ppm (5mg in 5g diet) recombinant avidin for 48 hours (see Figure 2.2 for photographs of the dissected components).

Avidin was readily detected in all of the components of the *M. brassicae* larvae (Figure 3.18), even after extensive washing with distilled water. As observed in aphids, in contrast to the recombinant avidin added to the diet (20kDa), the avidin in the lepidopteran components shows signs of proteolytic degradation (19kDa) and

dimerisation (36kDa). These results suggest that recombinant avidin travels throughout lepidopteran larvae, including parts of the insect only accessible via the haemolymph.

(a)



(b)

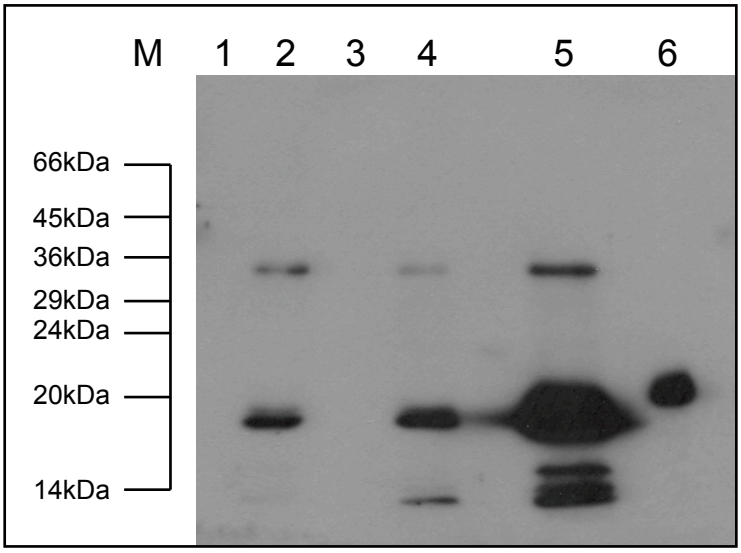


Figure 3.18.

Western analysis of dissected *M. brassicae* larvae after feeding on 1000ppm recombinant avidin for 48 hours.

One second exposures of the membranes transferred from 15% SDS-PAGE gels, probed with anti-avidin antibodies (1:10000) are shown. M is SDS 7 molecular weight marker. (a) Lane 1 is foregut from larvae fed with control diet. Lane 2 is foregut from larvae fed with recombinant avidin. Lane 3 is midgut from larvae fed with control diet. Lane 4 is midgut from larvae fed with recombinant avidin. Lane 5 is hindgut from larvae fed with control diet. Lane 6 is hindgut from larvae fed with recombinant avidin. Lane 7 is nerve cord from larvae fed with control diet. Lane 8 is nerve cord from larvae fed with recombinant avidin. Lane 9 is malphigian tubules from larvae fed with control diet. Lane 10 is malphigian tubules from larvae fed with recombinant avidin. (25µl loaded for each sample). All recombinant avidin samples show a slightly degraded avidin (19kDa) and some recombinant avidin dimerisation (36kDa). Lane 11 is 10ng of recombinant avidin (20kDa). (b) Lane 1 is fat bodies from larvae fed with control diet. Lane 2 is fat bodies from larvae fed with recombinant avidin. Lane 3 is gut contents from larvae fed with control diet. Lane 4 is gut contents from larvae fed with recombinant avidin. Lane 5 is faeces from larvae fed with recombinant avidin. (25µl loaded for each sample). All recombinant avidin samples show a slightly degraded avidin (19kDa) and some recombinant avidin dimerisation (36kDa). Lane 6 is 10ng of recombinant avidin (20kDa).

A recombinant avidin feeding timecourse experiment was carried out in lepidopteran larvae to establish how long avidin persists in the haemolymph (transport previously demonstrated in Hinchliffe, 2007). As in the previous experiment, 1000ppm (5mg in 5g diet) recombinant avidin was fed to fifth stadium *M. brassicae* larvae for 24 hours and then the larvae were chase-fed with control diet containing no avidin. Haemolymph samples were extracted at regular intervals throughout the feeding.

Avidin, showing signs of proteolytic degradation (19kDa), was detected in the haemolymph by western blotting (anti-avidin antibodies) after 20 minutes (Figure 3.19), suggesting that the transport process is relatively rapid. The levels of avidin remained fairly constant for the 24 hours feeding period, but once chase-fed, the avidin within the haemolymph was completely removed within 24 hours, suggesting that avidin reaches its final destination within the larvae in 24 hours.

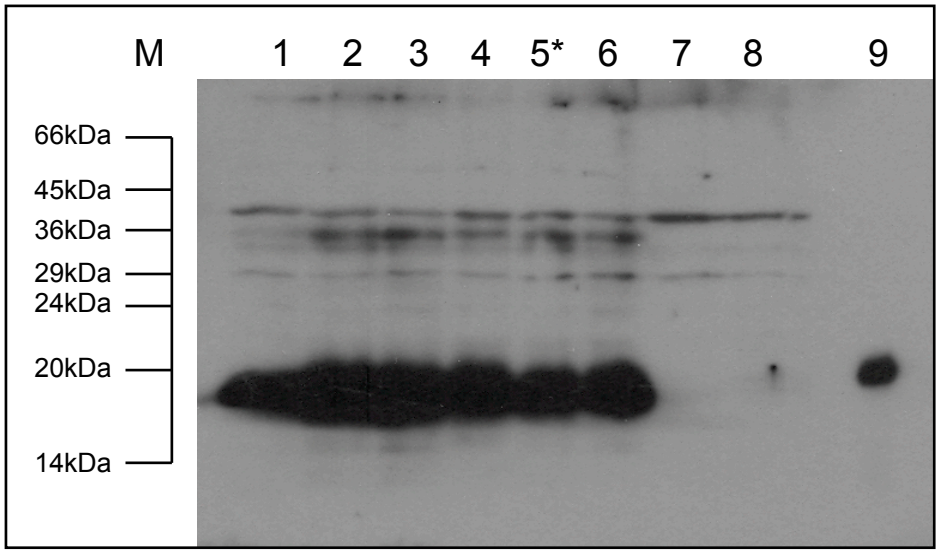


Figure 3.19.

Western analysis of *M. brassicae* haemolymph samples taken whilst feeding on 1000ppm recombinant avidin for 24 hours and chasing recombinant avidin with control diet for 48 hours.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is haemolymph from larvae fed on recombinant avidin for 20 minutes. Lane 2 is haemolymph from larvae fed on recombinant avidin for 1 hour. Lane 3 is haemolymph from larvae fed on recombinant avidin for 2 hours. Lane 4 is haemolymph from larvae fed on recombinant avidin for 6 hours. Lane 5 is haemolymph from larvae fed on recombinant avidin for 24 hours (* denotes the removal of recombinant avidin diet). Lane 6 is haemolymph from larvae fed on recombinant avidin for 24 hours followed by control diet for 6 hours (30 hours total). All of the haemolymph samples containing recombinant avidin show a slightly degraded avidin (19kDa). Lane 7 is haemolymph from larvae fed on recombinant avidin for 24 hours followed by control diet for 24 hours (48 hours total). Lane 8 is haemolymph from larvae fed on recombinant avidin for 24 hours followed by control diet for 48 hours (72 hours total). (10μl loaded for each sample). Lane 9 is 10ng of recombinant avidin (20kDa).

Discussion

This chapter presents the experiments carried out with recombinant avidin produced in *Pichia pastoris* that were required to gain a greater understanding of its structure and functionality both *in vitro* and *in vivo*.

The indicated molecular weight of recombinant avidin on SDS-PAGE gels (20kDa) is significantly larger (approximately 4kDa) than that of commercially available egg-white avidin (16kDa), to a greater extent than would be accounted for by the presence of the nine amino acid N-terminal extension and the $(His)_6$ tag sequence added at the C-terminus (Appendix 1). The single N-glycosylation site in the amino acid sequence is utilised in egg-white avidin (Green, 1990) and the results here confirm it is used in recombinant avidin. Deglycosylation of recombinant avidin gives a polypeptide whose estimated molecular weight is in good agreement with the predicted value from the nucleotide sequence alone (16kDa, Figure 3.1). Core glycosylation plus the addition of extra mannose residues (hyperglycosylation) is carried out by *P. pastoris*, normally adding approximately 2kDa or more to the molecular weight of a polypeptide (Cregg *et al.*, 1993). In agreement with the present results, glycosylation of recombinant avidin expressed in *P. pastoris* has been reported previously (Schenk *et al.*, 2008), however, the glycosylation in *P. pastoris* differs from that observed in egg-white avidin (Green, 1990), but the carbohydrate side chain does not affect the functional properties of the protein (Nardone *et al.*, 1998).

The gel filtration profile (Figure 3.2) shows that recombinant avidin forms tetrameric molecules similar to egg-white avidin, as both samples ran at an indicated molecular weight of approximately 60kDa compared to standard proteins. Recombinant avidin is very slightly larger, due to the hyperglycosylation described above. The listed tetrameric size of commercial avidin is 66kDa (Budavari *et al.*, 1996), therefore, the results here are within the experimental error of the gel filtration Sephacryl S-200 matrix (±10kDa) (5kDa-250kDa separation).

The interaction with immobilised biotin (Figure 3.3) shows that recombinant avidin is biologically fully functional in terms of biotin binding.

Toxicity of avidin to insects from a wide range of orders has been demonstrated (Chapter 1), however, no studies have reported the effects on hemipteran insects. Here, recombinant avidin shows a dose-dependent toxicity to *A. pisum* (Figure 3.4). The dose response curve for recombinant avidin in these bioassays shows a sharp transition between no significant effect on survival and complete mortality, similar to the threshold effect described previously (Hinchliffe, 2007), with an estimated LC_{50} value of 0.14mg/ml (140ppm, 2.1μM) (Figure 3.5). This value is comparable to the level of biotin in the standard artificial diet (4μM, 0.001mg/ml; one molecule of avidin binds four molecules of biotin and thus the LC_{50} is a two-fold molar excess of avidin binding sites over biotin) and suggests that these insects are highly biotin dependent.

In contrast, recombinant avidin has no significant effects on the survival of *S. avenae*, even when incorporated at 2mg/ml (2000ppm, 30μM) in artificial diet (Figure 3.6). However, the aphids show signs of retarded growth, being 40% smaller than the controls (Figure 3.7). Both insect cultures were maintained on plants and thus have a similar biotin 'background.' Reports of immunity to the effects of avidin are rare. Kramer *et al.* (2000) reported that of 11 coleopteran species tested, only one, *Prostephanus truncates* (larger grain borer) (Coleoptera: Bostrichidae, Horn 1878), exhibited a tolerance for avidin, where 17% mortality was recorded at a dietary level of 1000ppm (1mg/ml, 15μM). Malone *et al.* (2002) reported that whilst avidin had detrimental effects on larvae of *Sitona Lepidus* (clover root weevil) (Coleoptera: Curculionidae, Gyllenhal 1834), it was ineffective against adults. Similarly, Markwick *et al.* (2001) found that avidin had a limited toxicity to larvae of *Planotortrix octo* (green-headed leaf roller) (Lepidoptera: Tortricidae, Dugdale 1990) at concentrations up to 100μg/ml (100ppm, 0.15μM). The failure of avidin to show insecticidal effects on some species has been ascribed to the availability of biotin sequestered into eggs and/or embryos by parental generations, a view supported by experiments in which the LC_{50} of avidin towards *Epiphyas postvittana* (light brown apple moth) (Lepidoptera: Tortricidae, Walker 1863) larvae was decreased over 10-fold as a result of feeding for five generations on biotin-free diet (Markwick *et al.*, 2001).

Some evidence for the degradation of avidin by aphids *in vivo* has been presented here (Figure 3.8). Proteolytic degradation was considered to explain the difference in insecticidal activity between *A. pisum* and *S. avenae*, as the gut pH of *S. avenae* is lower than that of *A. pisum* and, results have shown that *Aphis gossypii* (cotton-melon aphid) (Hemiptera: Aphididae, Glover 1877) contain cathepsin L-like cysteine protease activity in their gut (Deraison *et al.*, 2004) and a gut-expressed family of cathepsin B-like proteases have been identified in *A. pisum* (Rispe *et al.*, 2008). However, in this case, there was no difference in the amount of proteolytic degradation between *A. pisum* and *S. avenae* (Figure 3.8). Beyond the scope of the stability experiment, a little analysis was carried out on the recombinant avidin degradation products (19kDa and 15kDa). Samples of *A. pisum* and *S. avenae* protein extract from the stability experiment were analysed by western blotting using anti-his tag antibodies (1:1000). The recombinant avidin degradation products were not immunoreactive, suggesting that the C-terminal $(His)_6$ tag is cleaved (blank blot not shown). This would most likely account for the 19kDa degradation product (Figure 3.8), as the two added C-terminal amino acid residues and the $(His)_6$ tag are 1kDa in size (Appendix 1). Experiments in this study also demonstrate that recombinant avidin is glycosylated by the *P. pastoris* yeast expression host (see page 54). The observed size difference of recombinant avidin compared to deglycosylated recombinant avidin is approximately 4kDa (Figure 3.1). A speculation can therefore be made that it is likely that the 15kDa degradation product (a difference of 4kDa) is due to deglycosylation of the recombinant avidin. However, further experimentation would be required to confirm these.

The absence of toxicity of avidin towards *S. avenae* cannot be due to a lack of dependence on exogenous biotin because these aphids do not survive on diets containing no biotin or low levels of biotin (Figure 3.9), although they appear to be slightly less sensitive than *A. pisum* to sub-optimal biotin concentrations, as 100% mortality was recorded in *A. pisum* at all but the standard artificial diet concentration of 4μM and there was 45% survival of *S. avenae* fed artificial diet containing 3μM biotin. This 4μM biotin threshold is no surprise as the artificial diet has been developed to specifically contain all of the nutrients required to support the aphid through its lifecycle.

The symbiotic *Buchnera* bacteria in aphids have varying complements of the genes involved in the biotin biosynthesis pathway, depending on the aphid host species (BuchneraBase, http://www.buchnera.org). However, *B. aphidicola* APS in *A. pisum* has lost the capacity to synthesise biotin, having only three (*bioA*, *bioD* and *bioB*) of the five genes involved in biotin biosynthesis (*bioW*, *bioF*, *bioA*, *bioD* and *bioB*) (Figure 3.20). The requirement of *A. pisum* for dietary biotin is thus not surprising. Based on the bioassays carried out, the *Buchnera* symbiont in *S. avenae* (genome not available) cannot complement a biotin deficiency either. Amplification of the biotin synthesis operon from *S. avenae* provided confirmation that the *Buchnera* symbiont in this aphid species similarly lacks the full complement of genes necessary for '*de novo*' synthesis of biotin (Figure 3.11).

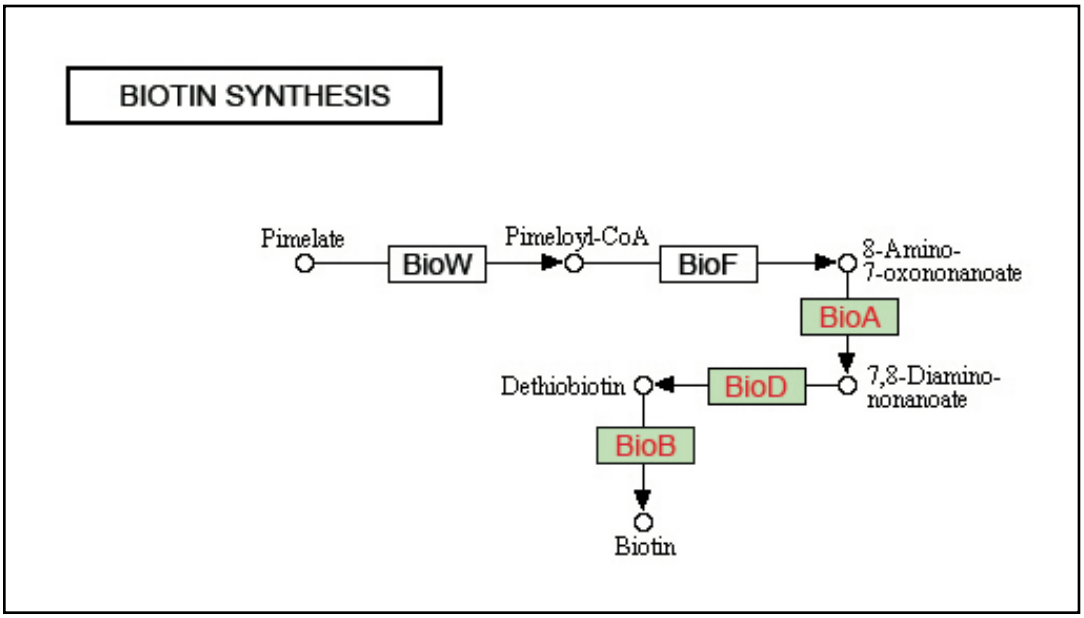


Figure 3.20.

Schematic representation of the biotin synthesis pathway.
(Adapted from KEGG database, http://www.genome.jp/kegg/kegg2.html).
The biotin synthesis genes present in *B. aphidicola* APS are highlighted in red.

The most surprising and significant result of the set of recombinant avidin toxicity experiments is the ability of *S. avenae*, but not *A. pisum*, to survive on biotin-free diets supplemented with recombinant avidin (Figure 3.10). The only plausible explanation for this is that biotin bound to the recombinant avidin can be recovered from the protein in a scavenging system. Recombinant avidin contains

biotin, as the culture medium in which the *P. pastoris* yeast is grown is supplemented with this vitamin (see Chapter 2, PTM1 trace salts). However, the biotin-binding sites on recombinant avidin cannot be saturated because the protein binds to immobilised biotin (Figure 3.3), but since tetrameric molecules are formed (Figure 3.2, discussed on page 85), if a small proportion of the binding sites contain biotin, the protein will show both functional activity and contain biotin. This is further supported by the aphid *in vivo* transport of recombinant avidin where, in both species of aphid (*S. avenae* and *A. pisum*), the protein is retained in the foregut (Figures 3.13, 3.14 and 3.15) and is detected in the insect even after a chase with non avidin-containing diet (Figure 3.15), showing that rapid elimination of avidin, which might prevent sequestration of ingested biotin, does not occur. So, in the present case, the data suggest that a combination of lower sensitivity to sub-optimal levels of biotin and a more effective system for scavenging biotin from recombinant avidin in the diet makes *S. avenae* relatively insensitive to the insecticidal effects of this protein when compared to *A. pisum*.

The attempts made here to isolate exactly what recombinant avidin is binding to in the gut were unsuccessful (Figures 3.17 and 3.18). This is perhaps no surprise as *in vitro* conditions will never fully represent the *in vivo* environment. It does however show that the avidin interaction is highly specific. The binding could be as a result of a direct interaction with the avidin or as an indirect interaction with biotin associated with the avidin. The saturation of recombinant avidin with biotin does not alleviate the binding in the gut, showing that the interaction is independent of avidin's biotin-binding capacity but this still does not exclude the possibility the binding is mediated through the associated biotin. One suggestion here may be that the avidin retention may arise as a result of binding to a complex of proteins be that directly, or indirectly via biotin. Any complex of proteins will have been separated during the gut protein extraction process and thus the specific binding site formed by the complex would be destroyed leading to the negative result seen in these experiments. One such protein complex would be the biotin transport system that is present in humans (Said, 2009). It is likely that aphids also have a biotin transport mechanism be it similar to that of humans or otherwise, but further investigation would be required to establish this.

The *in vivo* transport of recombinant avidin in lepidopteran larvae is a completely different scenario however. The protein is readily transported into the haemolymph and distributed around the insect following ingestion (Figures 3.19 and 3.20). The process of peptides crossing the lepidopteran gut epithelium is a common occurrence (Casartelli *et al.*, 2005; Fiandra *et al.*, 2009), although there is little data available as to the mechanism that the peptides use. However, in the present case, there appears to be different mechanisms for dealing with ingested avidin in hemiptera and lepidoptera.

In conclusion, the recombinant avidin produced in *P. pastoris* shows similar characteristics to the Sigma commercial alternative. The knowledge of the difference in avidin transport between hemiptera and lepidoptera will prove helpful and significant for the designing of insecticidal recombinant avidin fusion proteins and recombinant avidin conjugates (Chapters 4 and 5).

Chapter 4

Expression of Recombinant Avidin Fusion Proteins

Introduction

Results previously obtained as part of research towards an MSc thesis have shown that like snowdrop lectin (*Galanthus nivalis* agglutinin; GNA), avidin is transported from the gut contents to the haemolymph of lepidopteran larvae following oral delivery (Hinchliffe, 2007). Avidin therefore has the potential to act as a protein 'carrier' for the production of insecticidal fusion proteins, transporting fused toxins to their sites of action, thus playing a role similar to that of GNA in fusion proteins described previously (Fitches *et al.*, 2002, 2004; Pham-Trung *et al.*, 2006).

The production of synthetic avidin fusion proteins in *Escherichia coli* using glutathione S-transferase (GST) and hevein has been documented previously (Airenne and Kulomaa, 1995; Airenne *et al.*, 1999). However, there have not yet been any reports of synthetic avidin fusion proteins produced in *Pichia pastoris*. As functional avidin expresses with high yields in *P. pastoris* and purifies well (Hinchliffe, 2007), it is expected that an avidin fusion protein may behave similarly. The GNA fusion proteins expressed in *P. pastoris* (Fitches *et al.*, 2002, 2004; Pham-Trung *et al.*, 2006) provide an ideal starting point for the production of avidin fusion proteins: directly replacing the GNA with avidin.

The experiments in this chapter describe attempts to produce a fully functional, insecticidal avidin fusion protein in *P. pastoris*. Beginning with a fusion between avidin and the insecticidal ButaIT toxin from *Mesobuthus tamulus* (Indian red scorpion) (Scorpiones: Buthidae, Fabricius 1798), N-terminal and C-terminal variants will be produced. Peptide linker regions incorporating different restriction enzyme sites, an IgG Hinge successfully demonstrated by Airenne and Kulomaa (1995), and a fragment of GNA will also be investigated. Fusion proteins produced with the insecticidal Omega Atracotoxin (ωACTXHv1a) from *Hadronyche versuta*

(Australian Funnel Web Spider) (Araneae: Hexathelidae, Rainbow 1914) and the non-toxin alternative of_enhanced Green Fluorescent Protein (eGFP) will also be analysed. Finally, following computational and sequence analysis, structural mutations will be used to gain a greater understanding of the fusion proteins and synthetic fusion protein technology as a whole.

Using the evidence that avidin transports to the haemolymph of *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae from Hinchliffe (2007), the initial focus of the fusion protein testing will be on lepidopteran larvae (a major worldwide pest).

A successfully active fusion protein would have the potential to be developed as a commercial biopesticide pellet or spray, or eventually produced in a transgenic plant.

Results

Due to the number of fusion proteins produced and the large amount of data generated, Table 4.1 sumarises the main results of this chapter.

Fusion Protein	Linker	Details
ButaIT-Avidin 1	VDA (*Sal*I)	No activity upon injection. Degraded in gut following oral delivery.
ButaIT-Avidin 2	AAAA (*Not*I)	No activity upon injection. Degraded in gut following oral delivery.
Avidin-ButaIT 3	AAA (*Not*I)	No activity upon injection. Degraded in gut following oral delivery.
ButaIT-IgG Hinge-Avidin	IgG Hinge	No activity upon injection. Stable in haemolymph following injection.
Avidin-IgG Hinge-ButaIT	IgG Hinge	No activity upon injection. Stable in haemolymph following injection.
ωACTXHv1a-Avidin	AAAA (*Not*I)	No activity upon injection.
eGFP-Avidin	AAAA (*Not*I)	Degraded during purification. Appeared not active.
ButaIT-Gavidin	GNA Fragment	Appeared active upon injection and oral delivery. Degraded *in vivo* following oral delivery. (No activity upon injection following reassessment)
ButaIT-Gavidin 1 Mutation	GNA Fragment	Appeared less active upon injection. No activity following oral delivery. Degraded *in vivo* following oral delivery.
ButaIT-Gavidin 3 Mutations	GNA Fragment	No activity upon injection and oral delivery.
ButaIT-GNA Fragment	AAA (*Not*I)	Active upon injection. No activity following oral delivery.
GNA Fragment-Avidin	VDA (*Sal*I)	Active upon injection. No activity following oral delivery.

Table 4.1.

Summary of the fusion proteins produced.

Avidin-ButaIT Fusion Proteins

To assess the suitability of avidin for use as a 'carrier' in synthetic fusion protein technology, recombinant fusion proteins containing the insecticidal ButaIT toxin from *Mesobuthus tamulus* (Indian red scorpion) (Scorpiones: Buthidae, Fabricius 1798) (Chapter 1) and avidin were produced.

1. Production of the Avidin-ButaIT Fusion Protein Expression Constructs

Expression constructs for three ButaIT fusion proteins (designated ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3) were designed and produced by the cloning strategy described in Chapter 2. The ButaIT-Avidin 1 construct contained ButaIT toxin fused N-terminally to avidin using a *Sal*I restriction site linker (amino acid sequence: VDA). The expression construct also had a C-terminal extension encoding a $(His)_6$ tag. The ButaIT-Avidin 2 construct contained ButaIT toxin fused N-terminally to avidin using a *Not*I restriction site linker (amino acid sequence: AAAA). The Avidin-ButaIT 3 construct contained ButaIT toxin fused C-terminally to avidin using a *Not*I restriction site linker (amino acid sequence: AAA). The expression construct also had a C-terminal extension encoding a $(His)_6$ tag. The complete expression constructs of the three fusion proteins were cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequences, deduced amino acid sequences and schematic diagrams of the expression constructs are shown in Figures 4.1, 4.2 and 4.3.

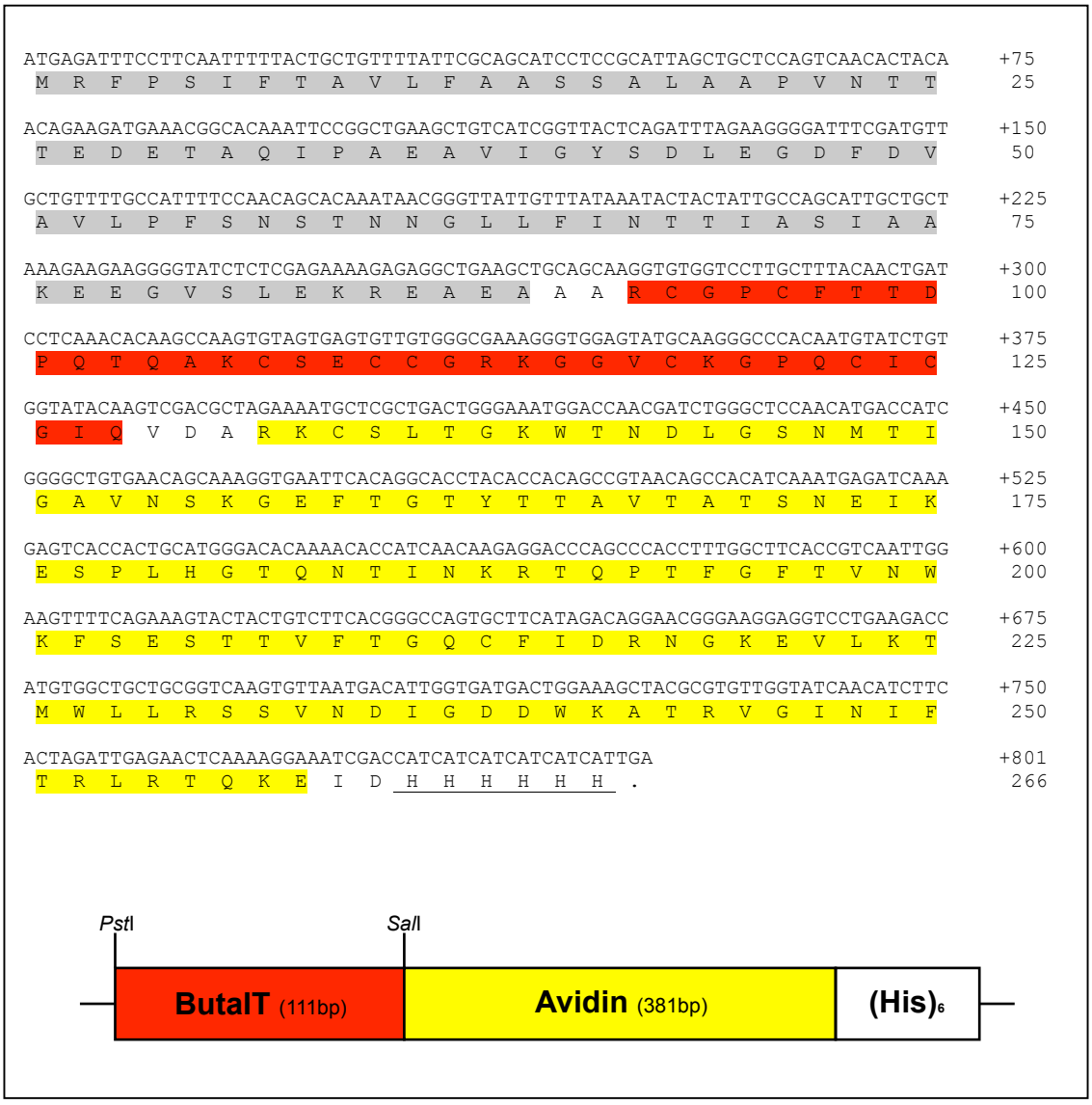


Figure 4.1.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ButaIT-Avidin 1 fusion protein - 'ButaIT (VDA) Avidin $(His)_6$.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted in red. The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black. Diagram not to scale.

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ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA     +75
 M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T      25

ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT     +150
 T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V      50

GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT     +225
 A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A  A      75

AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCAAGGTGTGGTCCTTGCTTTACAACTGAT     +300
 K  E  E  G  V  S  L  E  K  R  E  A  E  A  A  A  R  C  G  P  C  F  T  T  D      100

CCTCAAACACAAGCCAAGTGTAGTGAGTGTTGTGGGCGAAAGGGTGGAGTATGCAAGGGCCCACAATGTATCTGT     +375
 P  Q  T  Q  A  K  C  S  E  C  C  G  R  K  G  G  V  C  K  G  P  Q  C  I  C      125

GGTATACAAGCGGCCGCCGCTAGAAAATGCTCGCTGACTGGGAAATGGACCAACGATCTGGGCTCCAACATGACC     +450
 G  I  Q  A  A  A  A  R  K  C  S  L  T  G  K  W  T  N  D  L  G  S  N  M  T      150

ATCGGGGCTGTGAACAGCAAAGGTGAATTCACAGGCACCTACACCACAGCCGTAACAGCCACATCAAATGAGATC     +525
 I  G  A  V  N  S  K  G  E  F  T  G  T  Y  T  T  A  V  T  A  T  S  N  E  I      175

AAAGAGTCACCACTGCATGGGACACAAAACACCATCAACAAGAGGACCCAGCCCACCTTTGGCTTCACCGTCAAT     +600
 K  E  S  P  L  H  G  T  Q  N  T  I  N  K  R  T  Q  P  T  F  G  F  T  V  N      200

TGGAAGTTTTCAGAAAGTACTACTGTCTTCACGGGCCAGTGCTTCATAGACAGGAACGGGAAGGAGGTCCTGAAG     +675
 W  K  F  S  E  S  T  T  V  F  T  G  Q  C  F  I  D  R  N  G  K  E  V  L  K      225

ACCATGTGGCTGCTGCGGTCAAGTGTTAATGACATTGGTGATGACTGGAAAGCTACGCGTGTTGGTATCAACATC     +750
 T  M  W  L  L  R  S  S  V  N  D  I  G  D  D  W  K  A  T  R  V  G  I  N  I      250

TTCACTAGATTGAGAACTCAAAAGGAATGA                                                   +780
 F  T  R  L  R  T  Q  K  E  .                                                     259
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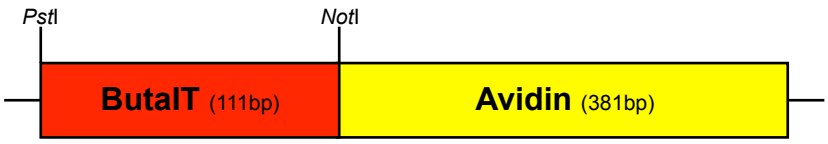


Figure 4.2.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ButaIT-Avidin 2 fusion protein - 'ButaIT (AAAA) Avidin.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted in red. The avidin sequence is highlighted in yellow. Diagram not to scale.

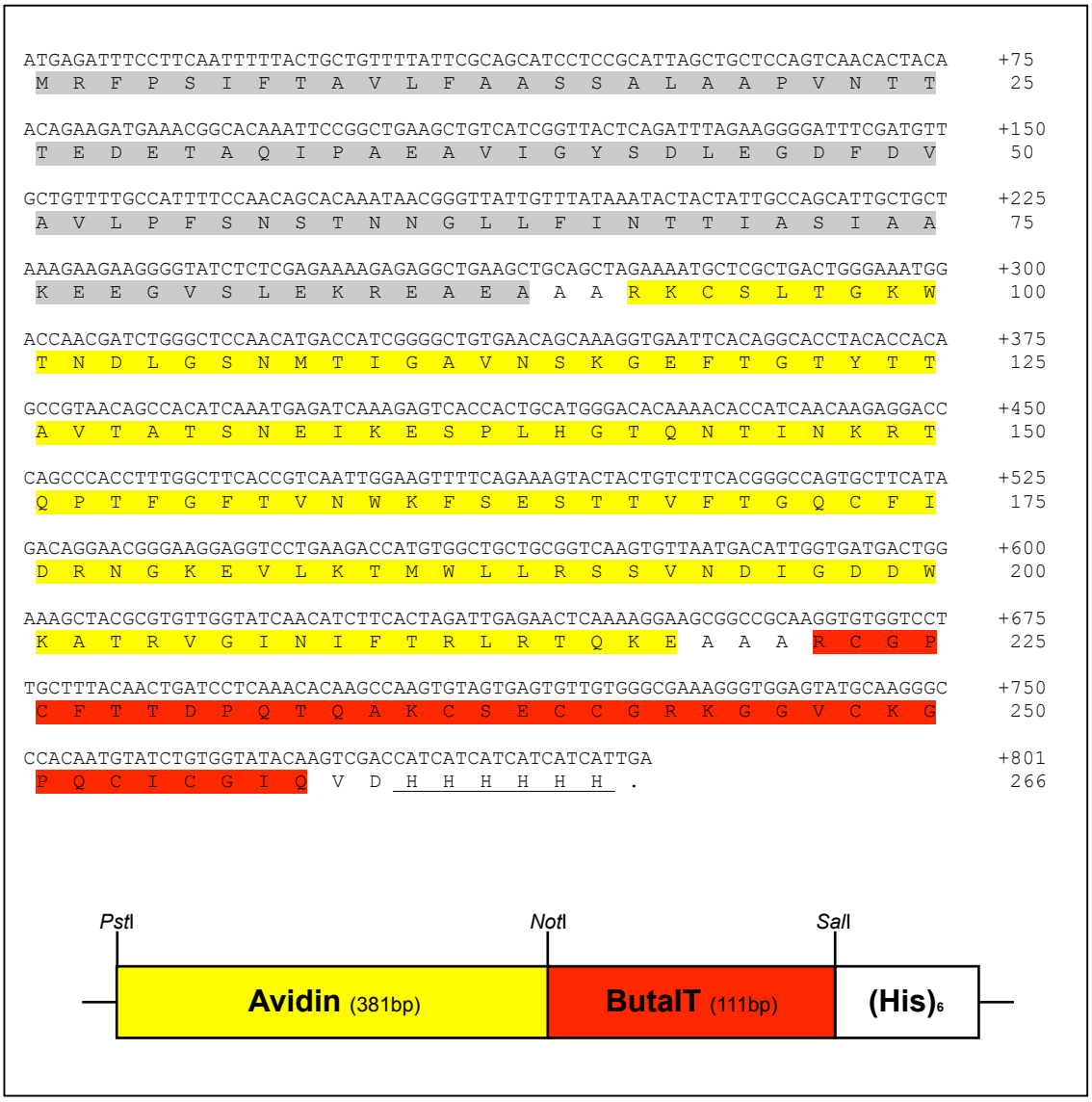


Figure 4.3.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the Avidin-ButaIT 3 fusion protein - 'Avidin (AAA) ButaIT $(His)_6$.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The avidin sequence is highlighted in yellow. The ButaIT toxin sequence is highlighted in red. The $(His)_6$ tag is underlined in black. Diagram not to scale.

2. Recombinant Expression of the Avidin-ButaIT Fusion Proteins

The fusion protein expression construct vectors were transformed into protease-deficient *Pichia pastoris*. Clones expressing the fusion proteins were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. High expressers were cultured separately in bench-top fermenters.

Recombinant ButaIT-Avidin 1 and Avidin-ButaIT 3 were both purified from culture supernatant by nickel affinity chromatography utilising the added $(His)_6$ tag. Recombinant ButaIT-Avidin 2 was purified from culture supernatant by SP-Sepharose ion exchange chromatography as no $(His)_6$ tag was present. Typical elution profiles of purifications by nickel affinity and SP-Sepharose column chromatography are shown in Figures 4.4 and 4.5.

From the amino acid sequences, the predicted sizes of ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3 are 19.4kDa, 18.4kDa and 19.4kDa respectively. The presence of ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3 was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluates were also analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4.6).

Recombinant ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3 were present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequences (23kDa, 22kDa and 23kDa respectively) (Figure 4.6(a)(ii), (b)(ii) and (c)(ii)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The SDS-PAGE analysis showed that there was a small amount of proteolytic degradation of all three of the purified fusion proteins (21kDa, 20kDa and 19kDa), with Avidin-ButaIT 3 showing the most evidence of proteolysis (Figure 4.6(a)(i), (b)(i) and (c)(i)). However, the column eluates contained a majority of the intact fusion protein.

Once analysed, column eluates were dialysed to remove salts and lyophilised by freeze-drying. Lyophilised proteins were quantified by SDS-PAGE. A typical quantification gel is shown in Figure 4.7. Yields of approximately 10mg of fusion

protein per litre of culture supernatant were obtained for all three recombinant fusion proteins.

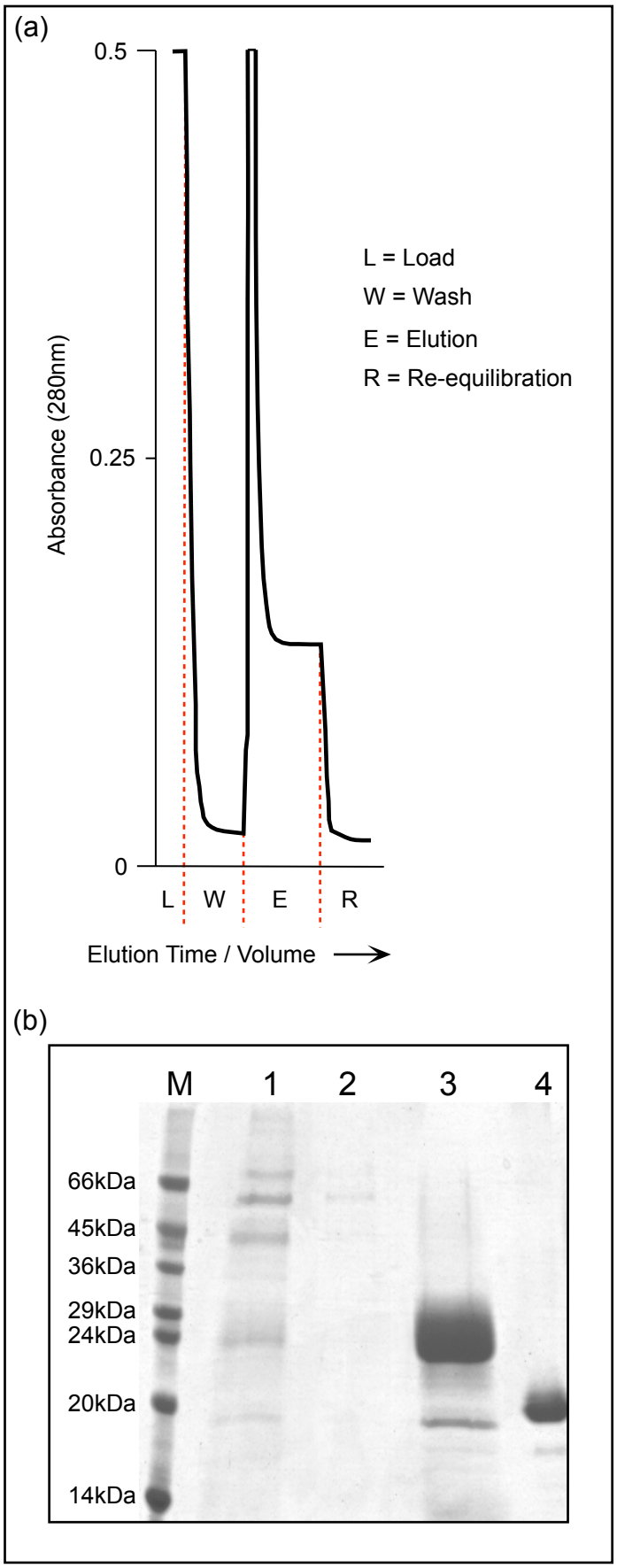


Figure 4.4.

Nickel column purification.

(a) Typical purification profile. One elution peak (E) was collected (10ml-15ml).

(b) Typical 15% SDS-PAGE analysis of a nickel column purification. M is SDS7 molecular weight marker. Lane 1 is nickel column load (L). Lane 2 is nickel column wash (W). Lane 3 is nickel column elution (E). (25µl loaded for each sample). Lane 4 is 5µg of recombinant avidin (20kDa).

(In this case, the fusion protein was Avidin-ButaIT 3).

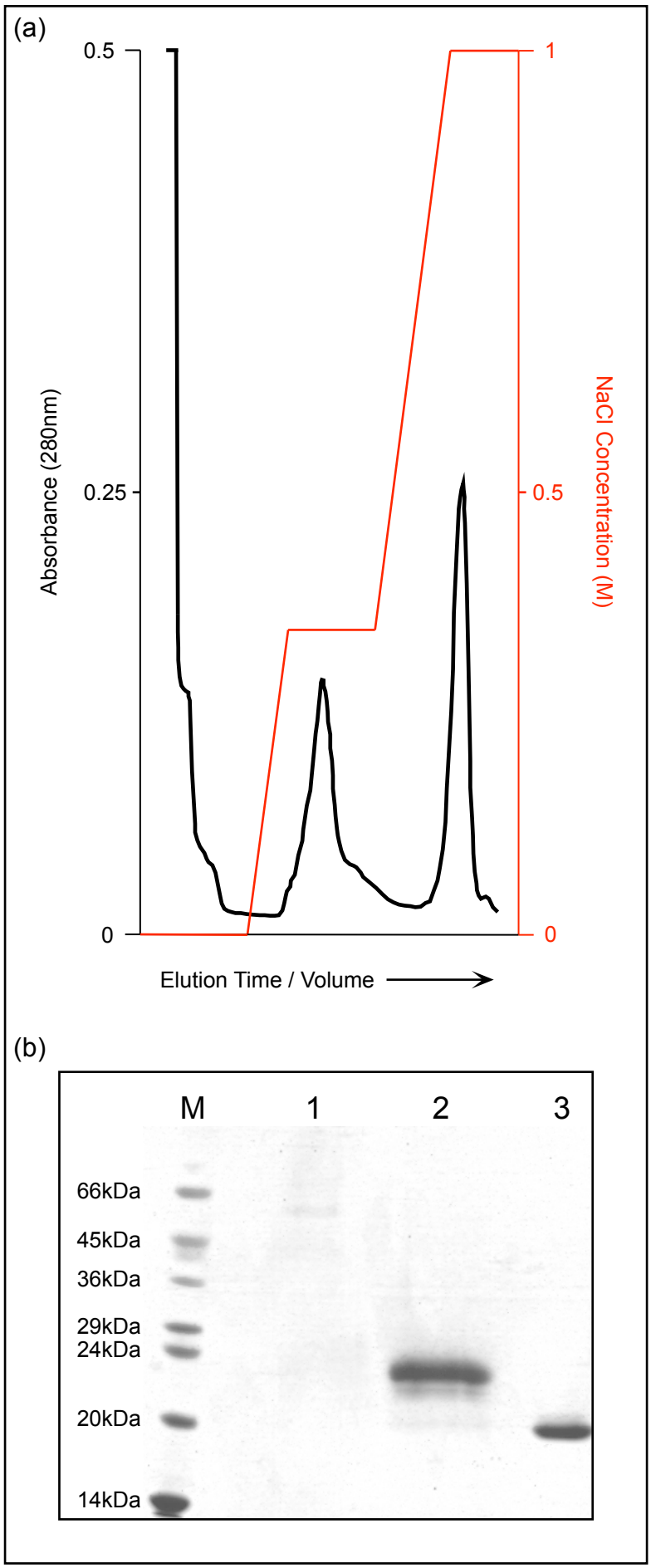
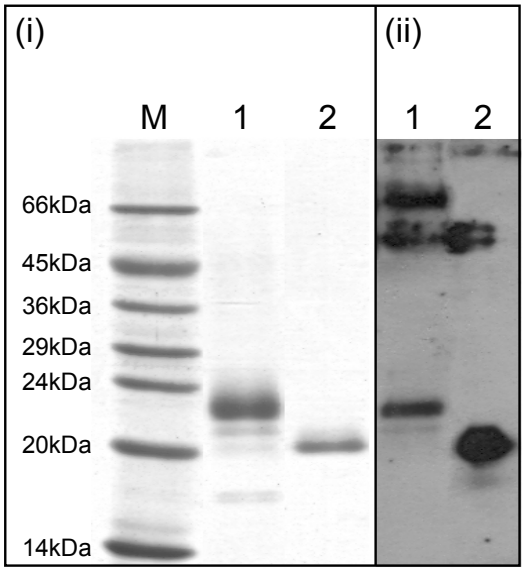


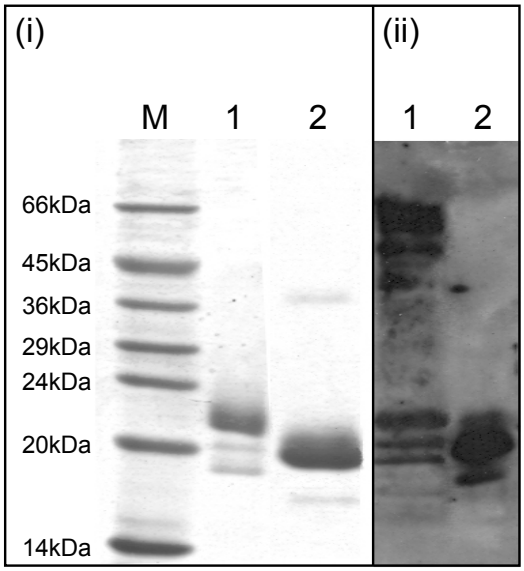
Figure 4.5.

SP-Sepharose column purification.
(a) Typical purification profile. As indicated by the red NaCl concentration line, the NaCl gradient was held at 0.35M to allow elution of protein through the complete column volume. Typically, the 0.35M NaCl elution peak contained the majority of the larger molecular weight proteins associated with *P. pastoris* yeast. Fusion proteins were present in the 1M NaCl elution peak.
(b) Typical 15% SDS-PAGE analysis of an SP-Sepharose column purification. M is SDS7 molecular weight marker. Lane 1 is 0.35M NaCl elution peak. Lane 2 is 1M NaCl elution peak. (25µl loaded for each sample). Lane 3 is 5µg of recombinant avidin (20kDa).
(In this case, the fusion protein was ButaIT-Avidin 2).

(a) ButaIT-Avidin 1



(b) ButaIT-Avidin 2



(c) Avidin-ButaIT 3

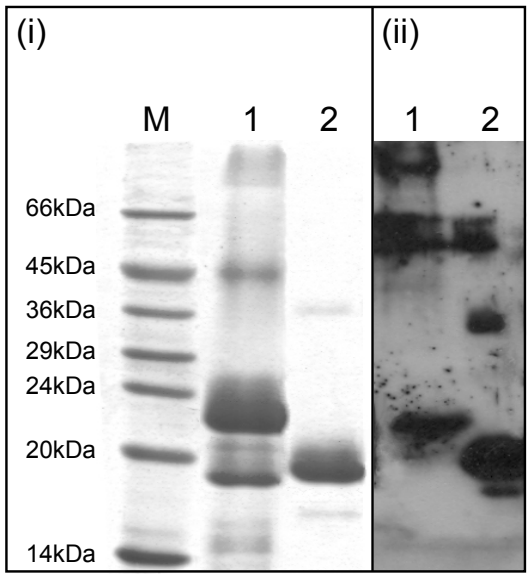


Figure 4.6.

SDS-PAGE and western analysis of the recombinant Avidin-ButaIT fusion proteins produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) ButaIT-Avidin 1: (i) 15% SDS-PAGE analysis. Lane 1 is 15µl of nickel column elution, showing ButaIT-Avidin 1 (23kDa) with slight proteolytic degradation (21kDa). Lane 2 is 2µg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified ButaIT-Avidin 1 (23kDa) with high molecular weight yeast proteins. Lane 2 is 20ng of recombinant avidin (20kDa). (b) ButaIT-Avidin 2: (i) 15% SDS-PAGE analysis. Lane 1 is 25µl of SP-Sepharose column elution, showing ButaIT-Avidin 2 (22kDa) with slight proteolytic degradation (20kDa and 19kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified ButaIT-Avidin 2 (22kDa) with high molecular weight yeast proteins and proteolytic degradation products (20kDa and 19kDa). Lane 2 is 20ng of recombinant avidin (20kDa). (c) Avidin-ButaIT 3: (i) 15% SDS-PAGE analysis. Lane 1 is 20µl of nickel column elution, showing Avidin-ButaIT 3(23kDa) with proteolytic degradation (19kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified Avidin-ButaIT 3 (23kDa) with high molecular weight yeast proteins. Lane 2 is 20ng of recombinant avidin (20kDa).

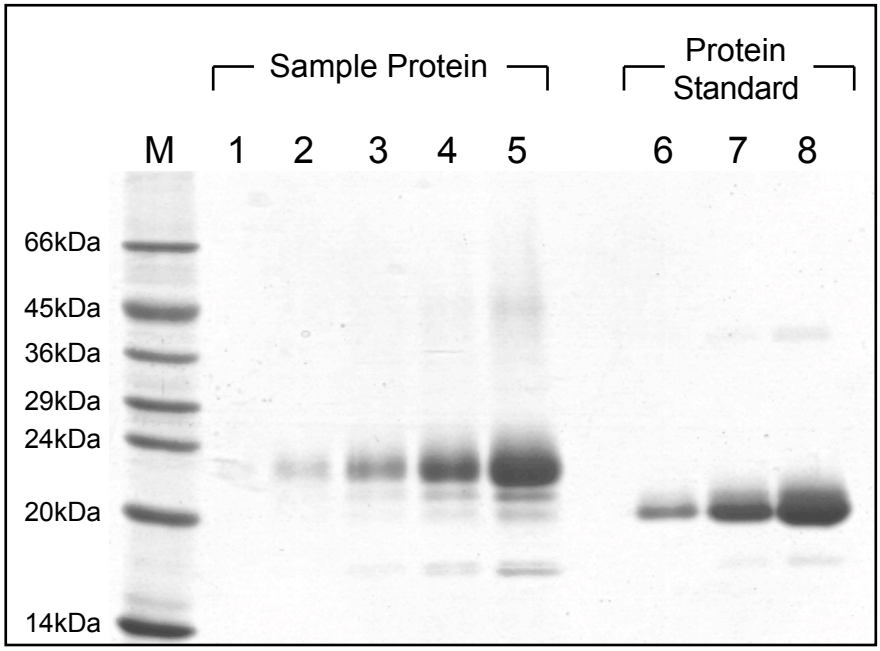


Figure 4.7.

15% SDS-PAGE Quantification Gel.

M is SDS7 molecular weight marker. Lanes 1-5 are 0.5μg, 1μg, 2.5μg, 5μg, and 10μg of sample protein respectively (in this case, ButaIT-Avidin 1). Lanes 6-8 are 1μg, 2.5μg and 5μg of protein standard respectively (in this case, recombinant avidin). Protein samples are quantified based on the content of the required protein, excluding any degradation products. In this case, the sample protein was deemed to contain 40% fusion protein.

3. Insecticidal Activity of the Recombinant Avidin-ButaIT Fusion Proteins

The recombinant Avidin-ButaIT fusion proteins were tested for insecticidal activity by injection into newly moulted fifth stadium *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae. Initially, to establish the amounts of fusion protein to include and for a comparison of activity, 10μg-150μg amounts of recombinant ButaIT toxin and recombinant ButaIT-GNA were injected. Recombinant ButaIT toxin had been prepared in *P. pastoris* by Dr Nanasaheb Chougule. Dr Elaine Fitches supplied both recombinant ButaIT and recombinant ButaIT-GNA proteins.

When injected into fifth stadium *M. brassicae* larvae, the recombinant ButaIT toxin showed a dose-dependent insecticidal activity over 24 hours, with complete mortality observed at a dose of 150μg, 50% mortality at a dose of 100μg and no

mortality at a dose of 50μg (Figure 4.8). Recombinant ButaIT-GNA also had a dose-dependent insecticidal activity, with 100% mortality observed at a dose of 50μg, 80% mortality at a dose of 25μg, 20% mortality at a dose of 20μg and no mortality at a dose of 10μg (Figure 4.8).

Based on these results, each 5μl injection contained a total of either 50μg or 100μg of recombinant Avidin-ButaIT fusion protein (quantified by SDS-PAGE) re-suspended in PBS. At those amounts, if the fusion protein activity was comparable to ButaIT-GNA, or if the toxin component of the fusion protein was active, mortality should be observed. Injections of PBS were also carried out as a negative control.

None of the recombinant Avidin-ButaIT fusion proteins were found to have any insecticidal activity in *M. brassicae* larvae (Figure 4.8). Injected larvae were completely unaffected by the fusion proteins, with 100% survival and no obvious visual or developmental effects being observed. Similar survival was observed in the PBS negative control injections (Figure 4.8). Even when the recombinant Avidin-ButaIT fusion proteins were injected at a dose of 100μg, no mortality was observed, suggesting that the ButaIT toxin component of the fusion protein is not active, most likely due to incorrect folding.

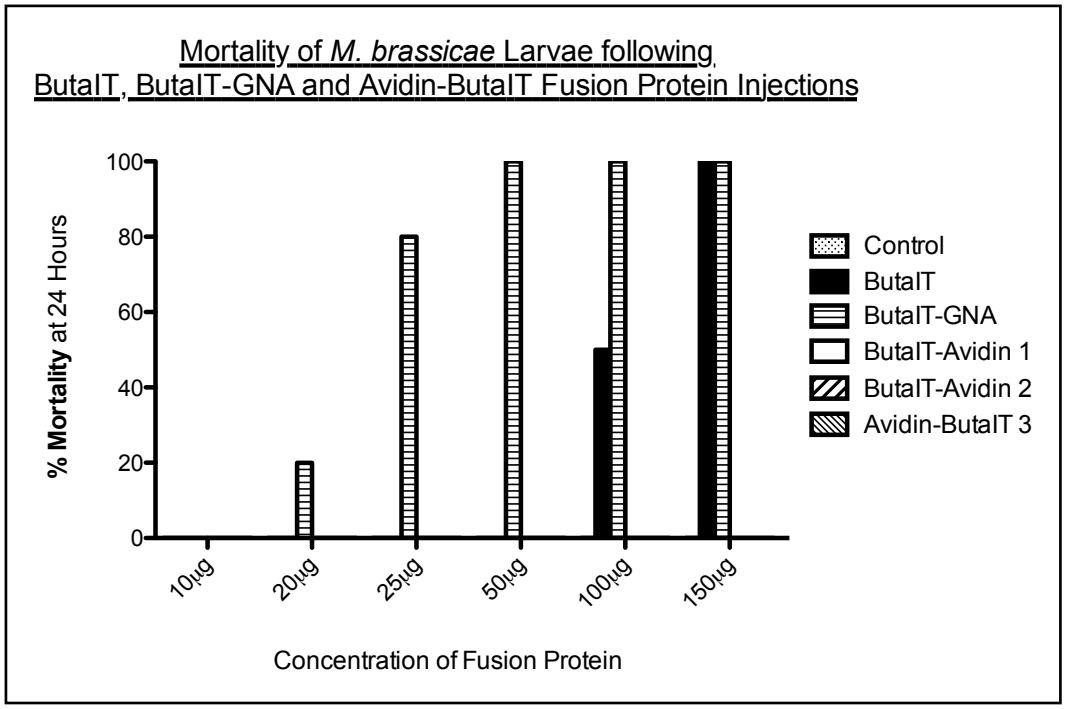


Figure 4.8.

Mortality of *M. brassicae* (cabbage moth) larvae following injection with varying amounts of recombinant ButaIT, ButaIT-GNA or the Avidin-ButaIT fusion proteins.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

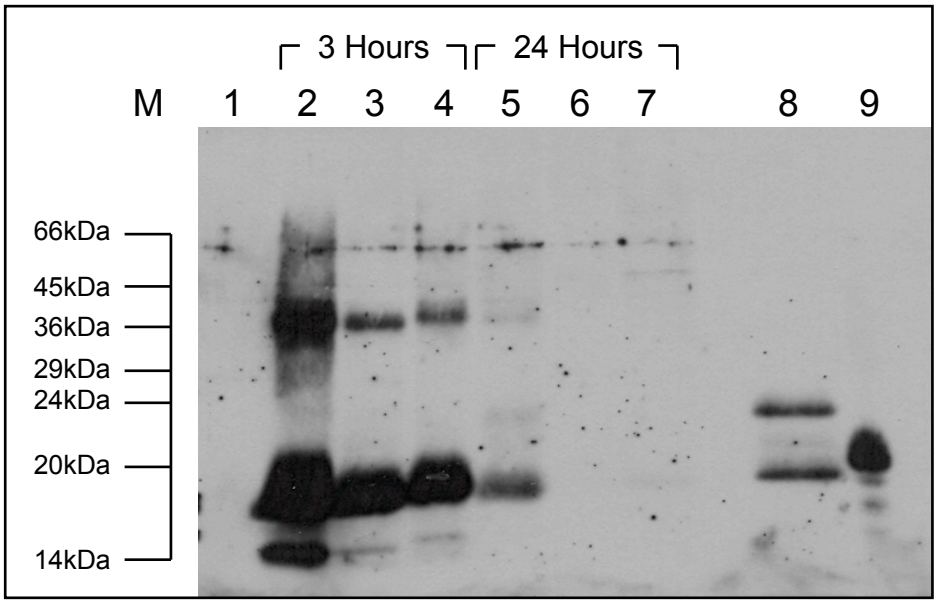
4. Analysis of the Recombinant Avidin-ButaIT Fusion Proteins

To investigate the *in vivo* transport of the recombinant Avidin-ButaIT fusion proteins, starved fifth stadium *M. brassicae* larvae were fed 5µl droplets containing 50µg of the fusion protein. Control larvae were fed droplets of phosphate buffered saline (PBS). Guts and haemolymph were extracted from five similarly fed larvae after three and 24 hours, pooled together and analysed by western blotting (anti-avidin antibodies) (Figure 4.9).

The results show that the fusion proteins were degraded in the gut of *M. brassicae* larvae. There was therefore no *in vivo* transport of the intact fusion proteins however, the remaining avidin (19kDa) was transported and readily detected in the haemolymph. The blots also show some recombinant avidin dimerisation (36kDa) (Figure 4.9).

The activity of avidin within the recombinant ButaIT fusion proteins was confirmed *in vitro* by incubation with a molar excess of immobilised biotin (agarose-biotin). The immobilised biotin matrix was mixed with 2.5μg of recombinant Avidin-ButaIT 3 for 30 minutes at room temperature. Following microcentrifugation, the pellet was washed twice with PBS. Finally, any bound recombinant avidin was eluted from the matrix by boiling. Analysis by SDS-PAGE (Figure 4.10) showed the absence of the fusion protein in the unbound fraction, an absence of protein in the wash fractions and 100% fusion protein eluted upon boiling. This shows that the avidin within the fusion protein was bound to the biotin matrix and suggests that it was fully functional.

(a) Guts



(b) Haemolymph

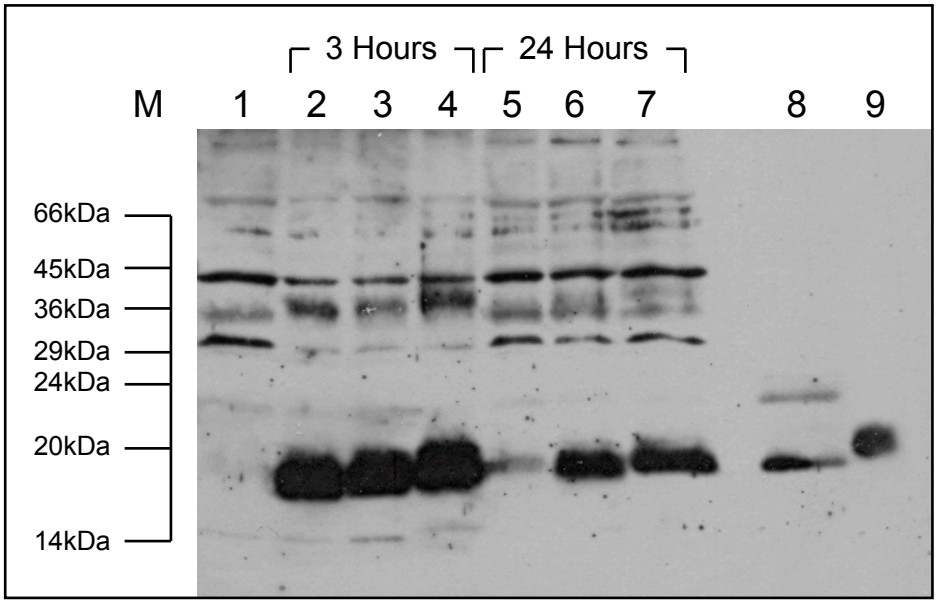


Figure 4.9.

Western analysis of *M. brassicae* larvae extracted guts and haemolymph, three hours and 24 hours after feeding on a 50µg recombinant Avidin-ButaIT fusion protein droplet.

Five second exposures of the membranes transferred from 15% SDS-PAGE gels, probed with anti-avidin antibodies (1:10000) are shown. M is SDS 7 molecular weight marker. (a) Guts. Lane 1 is guts from larvae fed with PBS (control). Lanes 2-4 are guts from larvae fed with ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3 for three hours respectively. All three samples contain avidin showing signs of proteolytic degradation (19kDa) and some recombinant avidin dimerisation (36kDa). Lanes 5-7 are guts from larvae fed with ButaIT-Avidin 1, ButaIT-Avidin 2 and Avidin-ButaIT 3 for 24 hours respectively. The amount of degraded avidin (19kDa) has decreased. (20µl loaded for each sample). Lane 8 is 10ng of Avidin-ButaIT 3 (23kDa), showing signs of proteolytic degradation (19kDa). Lane 9 is 10ng of recombinant avidin (20kDa). (b) Haemolymph. Lanes 1-9 are the equivalent haemolymph loadings. The avidin antibody shows some reactivity with other haemolymph proteins (Lane 1).

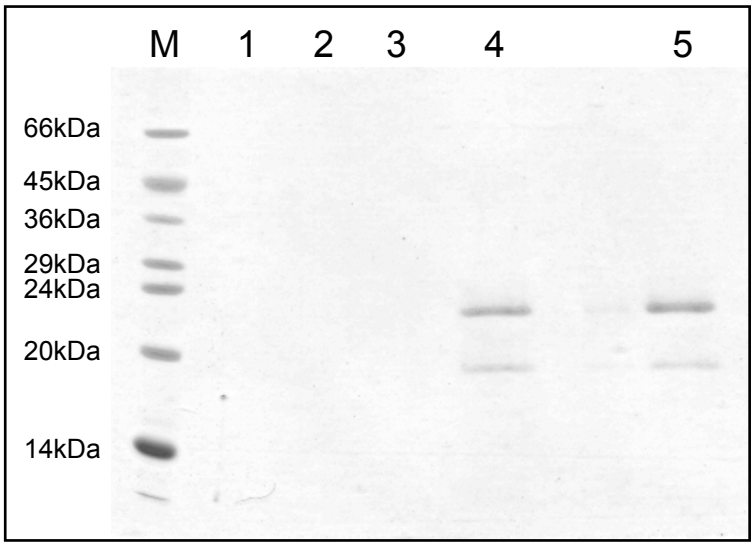


Figure 4.10.

15% SDS-PAGE analysis following *in vitro* binding of recombinant Avidin-ButaIT 3 to immobilised biotin (agarose-biotin).

M is SDS 7 molecular weight marker. Lane 1 is the unbound fraction. Lanes 2 and 3 are the PBS wash fractions. Lane 4 is the elution fraction, showing recombinant Avidin-ButaIT 3 (23kDa) with slight proteolytic degradation (19kDa). (25µl loaded for each sample). Lane 5 is 5µg of Avidin-ButaIT 3 (23kDa), showing signs of proteolytic degradation (19kDa).

IgG Hinge Avidin-ButaIT Fusion Proteins

To address the suggestion that the ButaIT toxin may have folded incorrectly, a linker region was incorporated into the fusion proteins to allow more space between the components.

1. Production of the IgG Hinge Avidin-ButaIT Fusion Protein Expression Constructs

Expression constructs for two further fusion proteins containing an Immunoglobulin G (IgG) hinge linker region were developed from the existing ButaIT-Avidin 2 and Avidin-ButaIT 3 fusion proteins (designated ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT) using the cloning strategy described in Chapter 2. The IgG hinge region (amino acid sequence: PKPSTPPGSS, optimised with yeast codons) was included to increase the spatial distribution between the ButaIT toxin and the avidin, hopefully giving less hindrance and thus allowing the ButaIT toxin to fold correctly. The successful use of the IgG hinge as a spacer in peptide fusions was previously demonstrated by Airenne and Kulomaa (1995). The complete expression constructs of the two fusion proteins were cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequences, deduced amino acid sequences and schematic diagrams of the expression constructs are shown in Figures 4.11 and 4.12.

```
ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA     +75
 M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T      25

ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT     +150
 T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V      50

GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT     +225
 A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A  A      75

AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCAAGGTGTGGTCCTTGCTTTACAACTGAT     +300
 K  E  E  G  V  S  L  E  K  R  E  A  E  A  A  A  R  C  G  P  C  F  T  T  D      100

CCTCAAACACAAGCCAAGTGTAGTGAGTGTTGTGGGCGAAAGGGTGGAGTATGCAAGGGCCCACAATGTATCTGT     +375
 P  Q  T  Q  A  K  C  S  E  C  C  G  R  K  G  G  V  C  K  G  P  Q  C  I  C      125

GGTATACAAGCGGCCGCACCAAAGCCAAGTACTCCACCAGGTAGTAGTGCGGCCGCCGCTAGAAAATGCTCGCTG     +450
 G  I  Q  A  A  A  P  K  P  S  T  P  P  G  S  S  A  A  A  A  R  K  C  S  L      150

ACTGGGAAATGGACCAACGATCTGGGCTCCAACATGACCATCGGGGCTGTGAACAGCAAAGGTGAATTCACAGGC     +525
 T  G  K  W  T  N  D  L  G  S  N  M  T  I  G  A  V  N  S  K  G  E  F  T  G      175

ACCTACACCACAGCCGTAACAGCCACATCAAATGAGATCAAAGAGTCACCACTGCATGGGACACAAAACACCATC     +600
 T  Y  T  T  A  V  T  A  T  S  N  E  I  K  E  S  P  L  H  G  T  Q  N  T  I      200

AACAAGAGGACCCAGCCCACCTTTGGCTTCACCGTCAATTGGAAGTTTTCAGAAAGTACTACTGTCTTCACGGGC     +675
 N  K  R  T  Q  P  T  F  G  F  T  V  N  W  K  F  S  E  S  T  T  V  F  T  G      225

CAGTGCTTCATAGACAGGAACGGGAAGGAGGTCCTGAAGACCATGTGGCTGCTGCGGTCAAGTGTTAATGACATT     +750
 Q  C  F  I  D  R  N  G  K  E  V  L  K  T  M  W  L  L  R  S  S  V  N  D  I      250

GGTGATGACTGGAAAGCTACGCGTGTTGGTATCAACATCTTCACTAGATTGAGAACTCAAAAGGAATGA           +819
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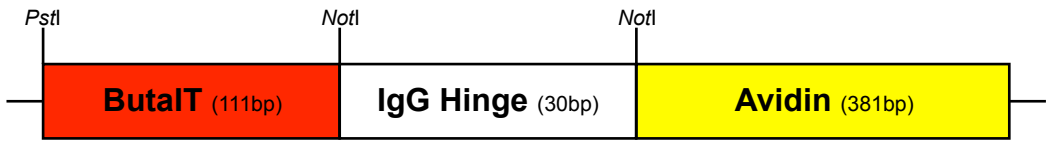

Figure 4.11.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ButaIT-IgG Hinge-Avidin fusion protein - 'ButaIT (IgG Hinge) Avidin.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted in red. The IgG Hinge linker is underlined in black. The avidin sequence is highlighted in yellow. Diagram not to scale.

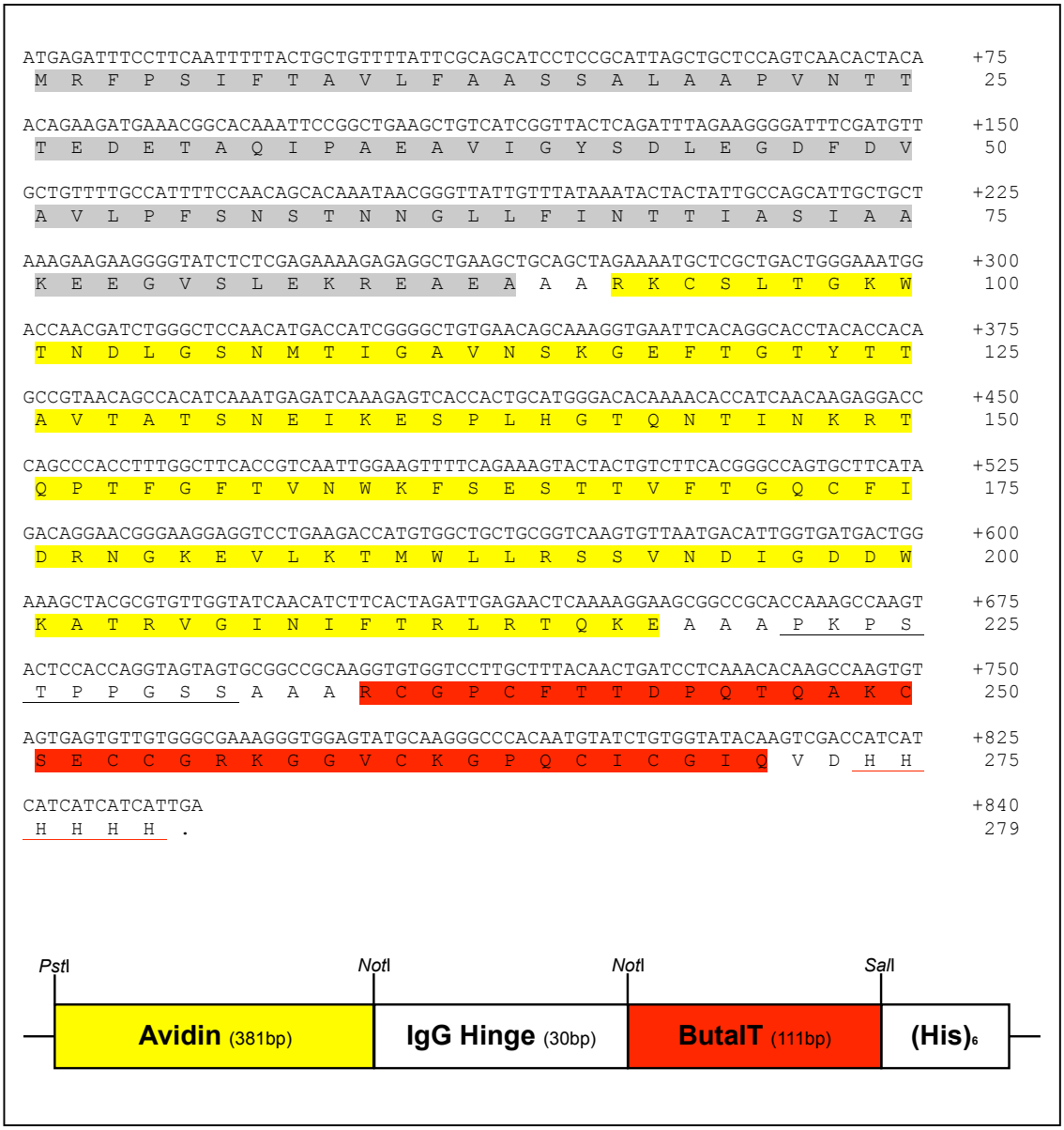

Figure 4.12.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the Avidin-IgG Hinge-ButaIT fusion protein - 'Avidin (IgG Hinge) ButaIT $(His)_6$.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The avidin sequence is highlighted in yellow. The IgG Hinge linker is underlined in black. The ButaIT toxin sequence is highlighted in red. The $(His)_6$ tag is underlined in red. Diagram not to scale.

2. Recombinant Expression of the IgG Hinge Avidin-ButaIT Fusion Proteins

The fusion protein expression construct vectors were transformed into protease-deficient *P. pastoris*. Clones expressing the fusion proteins were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. High expressers were cultured separately in bench-top fermenters.

Recombinant ButaIT-IgG Hinge-Avidin was purified from culture supernatant by SP-Sepharose ion exchange chromatography (Figure 4.5), whilst Avidin-IgG Hinge-ButaIT was purified from culture supernatant by nickel affinity chromatography utilising the added $(His)_6$ tag (Figure 4.4).

From the amino acid sequences, the predicted sizes of ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT are 19.5kDa and 20.5kDa respectively. The presence of ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluates were also analysed by SDS-PAGE (Figure 4.13).

Recombinant ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT were present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequences (23.5kDa) (Figure 4.13(a)(ii) and (b)(ii)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3).

Once analysed, column eluates were dialysed to remove salts and lyophilised by freeze-drying. Lyophilised proteins were quantified by SDS-PAGE (Figure 4.7). Yields of approximately 10mg of fusion protein per litre of culture supernatant were obtained for both recombinant fusion proteins.

(a) ButaIT-IgG Hinge-Avidin (b) Avidin-IgG Hinge-ButaIT

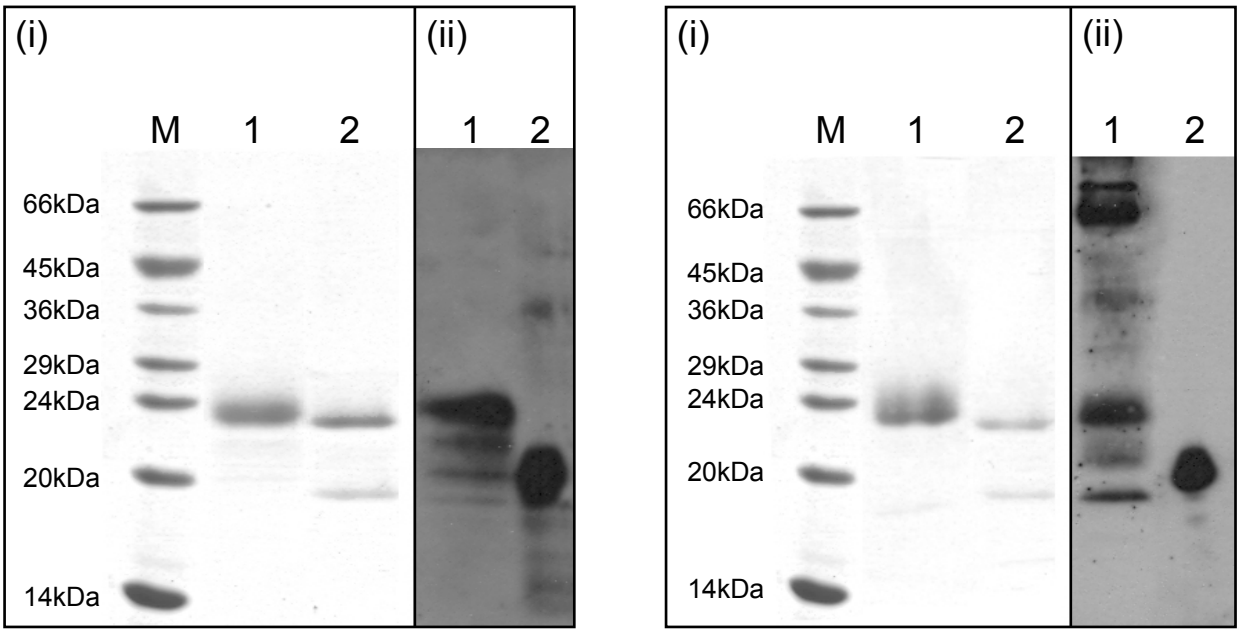

Figure 4.13.

SDS-PAGE and western analysis of the recombinant IgG Hinge Avidin-ButaIT fusion proteins produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) ButaIT-IgG Hinge-Avidin: (i) 15% SDS-PAGE analysis. Lane 1 is 25µl of SP-Sepharose column elution, showing ButaIT-IgG Hinge-Avidin (23.5kDa). Lane 2 is 2.5µg of Avidin-ButaIT 3 (23kDa), showing signs of proteolytic degradation (19kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified ButaIT-IgG Hinge Avidin (23.5kDa) with proteolytic degradation products (22kDa and 20.5kDa). Lane 2 is 10ng of recombinant avidin (20kDa). (b) Avidin-IgG Hinge-ButaIT: (i) 15% SDS-PAGE analysis. Lane 1 is 15µl of nickel column elution, showing Avidin-IgG Hinge-ButaIT (23.5kDa). Lane 2 is 2.5µg of Avidin-ButaIT 3 (23kDa), showing signs of proteolytic degradation (19kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified Avidin-IgG Hinge-ButaIT (23.5kDa) with proteolytic degradation products (22kDa and 19kDa) and high molecular weight yeast proteins. Lane 2 is 10ng of recombinant avidin (20kDa).

3. Insecticidal Activity of the Recombinant IgG Hinge Avidin-ButaIT Fusion Proteins

Neither of the recombinant IgG Hinge Avidin-ButaIT fusion proteins were found to have any insecticidal activity when assessed by injection into newly moulted fifth stadium *M. brassicae* larvae (Figure 4.14). Each 5µl injection contained a total of either 50µg or 100µg of fusion protein (quantified by SDS-

PAGE) re-suspended in PBS. Recombinant ButaIT-GNA was injected as a comparison and PBS was used as a negative control.

Injected *M. brassicae* larvae were completely unaffected by the recombinant IgG Hinge Avidin-ButaIT fusion proteins, with 100% survival and no obvious visual or developmental effects being observed. Recombinant ButaIT-GNA injected larvae showed 95% and 100% mortality at the two doses whilst the PBS negative control showed 100% survival (Figure 4.14). The absence of mortality at a 100μg dose of either recombinant IgG Hinge Avidin-ButaIT fusion protein suggests that the ButaIT toxin component of the fusion protein may still be incorrectly folded.

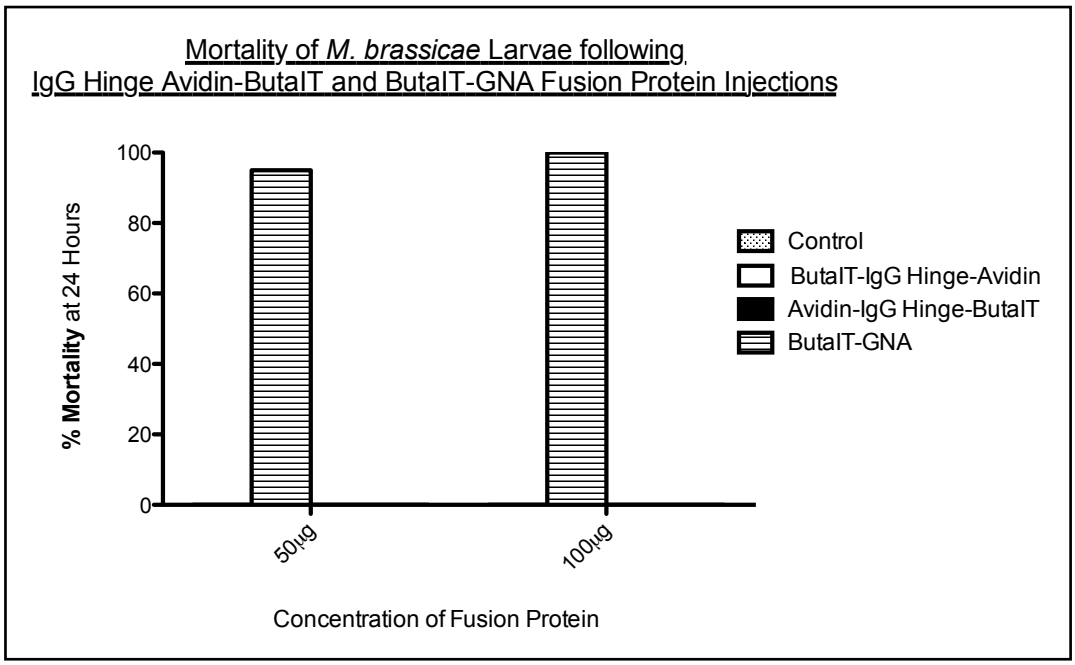

Figure 4.14.

Mortality of *M. brassicae* larvae following injection with varying amounts of recombinant IgG Hinge Avidin-ButaIT or ButaIT-GNA fusion proteins.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

4. Analysis of the Recombinant IgG Hinge Avidin-ButaIT Fusion Proteins

To evaluate the *in vivo* degradation of the recombinant IgG Hinge Avidin-ButaIT fusion proteins, newly moulted fifth stadium *M. brassicae* larvae were injected with 5μl of solution containing 50μg of the fusion protein. Control larvae were injected with PBS. Haemolymph was extracted from five larvae after 24 and 48

hours, pooled together and analysed by western blotting (anti-avidin antibodies) (Figure 4.15).

The result shows that the fusion proteins are stable in the haemolymph of *M. brassicae* larvae (23.5kDa) for at least 48 hours following injection, further supporting that the ButaIT toxin must be incorrectly folded.

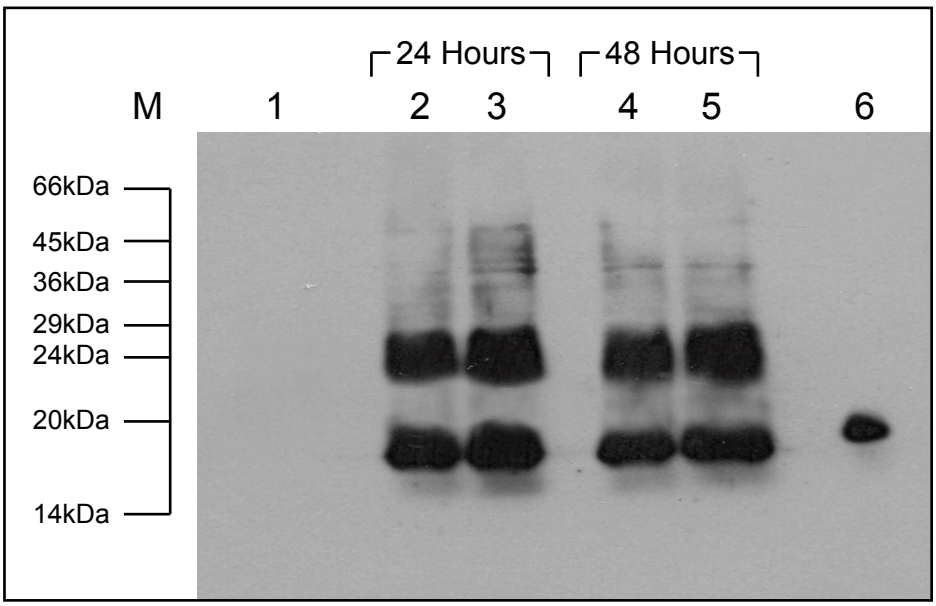

Figure 4.15.

Western analysis of *M. brassicae* larvae haemolymph 24 hours and 48 hours after injection of 50µg of recombinant IgG Hinge Avidin-ButaIT fusion protein.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is haemolymph from larvae injected with PBS (control). Lanes 2 and 3 are haemolymph from larvae 24 hours following injection with ButaIT-IgG Hinge-Avidin and Avidin-IgG Hinge-ButaIT respectively. (25µl loaded for each sample). Lanes 4 and 5 are the equivalent haemolymph loadings at 48 hours. All of the IgG Hinge Avidin-ButaIT fusion protein samples show both the fusion protein (23.5kDa) and a proteolytic degradation product (19kDa). Lane 6 is 10ng of recombinant avidin (20kDa).

ωACTXHv1a-Avidin (Omega Atracotoxin) Fusion Protein

To establish that the inactivity of the Avidin-ButaIT fusion proteins was not due to an incompatibility between ButaIT toxin and avidin, a different toxin was used.

1. Production of the ωACTXHv1a-Avidin Fusion Protein Expression Construct

An expression construct for a fusion protein incorporating the ωACTXHv1a toxin (Omega Atracotoxin) (Chapter 1) as a replacement for the ButaIT toxin was produced using the cloning strategy described in Chapter 2. The ωACTXHv1a-Avidin construct contained ωACTXHv1a toxin fused N-terminally to avidin using a *Not*I restriction site linker (amino acid sequence: AAAA). The complete fusion protein expression construct was cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequence, deduced amino acid sequence and schematic diagram of the expression construct is shown in Figure 4.16.

```
ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA     +75
 M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T      25

ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT     +150
 T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V      50

GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT     +225
 A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A  A      75

AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCATCTCCAACTTGTATTCCATCTGGTCAA     +300
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CCATGTCCATATAATGAAAATTGTTGTTCTCAATCTTGTACTTTTAAAGAAAATGAAAATGGTAATACTGTTAAA     +375
 P  C  P  Y  N  E  N  C  C  S  Q  S  C  T  F  K  E  N  E  N  G  N  T  V  K      125

AGATGTGATGCGGCCGCCGCTAGAAAATGCTCGCTGACTGGGAAATGGACCAACGATCTGGGCTCCAACATGACC     +450
 R  C  D  A  A  A  A  R  K  C  S  L  T  G  K  W  T  N  D  L  G  S  N  M  T      150

ATCGGGGCTGTGAACAGCAAAGGTGAATTCACAGGCACCTACACCACAGCCGTAACAGCCACATCAAATGAGATC     +525
 I  G  A  V  N  S  K  G  E  F  T  G  T  Y  T  T  A  V  T  A  T  S  N  E  I      175

AAAGAGTCACCACTGCATGGGACACAAAACACCATCAACAAGAGGACCCAGCCCACCTTTGGCTTCACCGTCAAT     +600
 K  E  S  P  L  H  G  T  Q  N  T  I  N  K  R  T  Q  P  T  F  G  F  T  V  N      200

TGGAAGTTTTCAGAAAGTACTACTGTCTTCACGGGCCAGTGCTTCATAGACAGGAACGGGAAGGAGGTCCTGAAG     +675
 W  K  F  S  E  S  T  T  V  F  T  G  Q  C  F  I  D  R  N  G  K  E  V  L  K      225

ACCATGTGGCTGCTGCGGTCAAGTGTTAATGACATTGGTGATGACTGGAAAGCTACGCGTGTTGGTATCAACATC     +750
 T  M  W  L  L  R  S  S  V  N  D  I  G  D  D  W  K  A  T  R  V  G  I  N  I      250

TTCACTAGATTGAGAACTCAAAAGGAATGA                                                   +780
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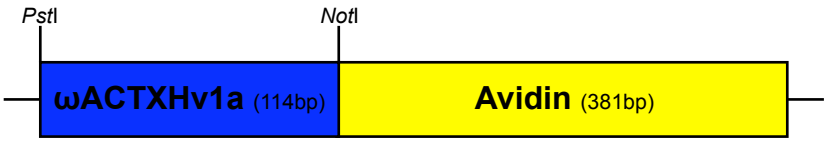

Figure 4.16.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ωACTXHv1a-Avidin fusion protein - 'ωACTXHv1a (AAAA) Avidin.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ωACTXHv1a toxin sequence is highlighted in blue. The avidin sequence is highlighted in yellow. Diagram not to scale.

2. Recombinant Expression of the ωACTXHv1a-Avidin Fusion Protein

The fusion protein expression construct vector was transformed into protease-deficient *P. pastoris*. Clones expressing the fusion protein were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. A high expresser was cultured in a bench-top fermenter.

Recombinant ωACTXHv1a-Avidin was purified from culture supernatant by SP-Sepharose ion exchange chromatography (Figure 4.5).

From the amino acid sequence, the predicted size of ωACTXHv1a-Avidin is 18.5kDa. The presence of ωACTXHv1a-Avidin was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluate was also analysed by SDS-PAGE (Figure 4.17).

Recombinant ωACTXHv1a-Avidin was present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequence (23kDa) (Figure 4.17(b)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The SDS-PAGE analysis showed that there was a small amount of proteolytic degradation of the purified fusion protein (19kDa) (Figure 4.17(a)). However, the column eluate contained a majority of the intact fusion protein.

Once analysed, column eluate was dialysed to remove salts and lyophilised by freeze-drying. Lyophilised protein was quantified by SDS-PAGE (Figure 4.7). A yield of approximately 10mg of fusion protein per litre of culture supernatant was obtained.

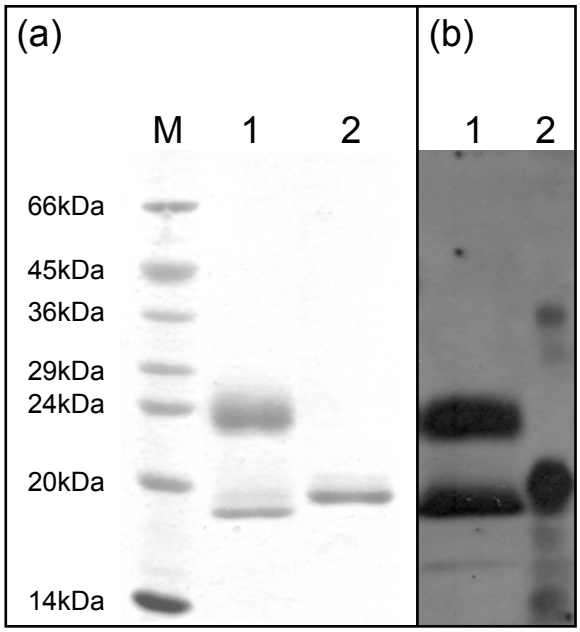

Figure 4.17.

SDS-PAGE and western analysis of the recombinant ωACTXHv1a-Avidin fusion protein produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) 15% SDS-PAGE analysis. Lane 1 is 25µl of SP-Sepharose column elution, showing ωACTXHv1a-Avidin (23kDa) with slight proteolytic degradation (19kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (b) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified ωACTXHv1a-Avidin (23kDa) and a proteolytic degradation product (19kDa). Lane 2 is 10ng of recombinant avidin (20kDa).

3. Insecticidal Activity of the Recombinant ωACTXHv1a-Avidin Fusion Protein

The recombinant ωACTXHv1a-Avidin fusion protein was tested for insecticidal activity by injection into newly moulted fifth stadium *M. brassicae* larvae. Initially, to establish the amount of fusion protein to include and for a comparison of activity, 10µg-150µg amounts of recombinant ωACTXHv1a-GNA were injected. Dr Elaine Fitches supplied recombinant ωACTXHv1a-GNA fusion protein.

When injected into fifth stadium *M. brassicae* larvae, the recombinant ωACTXHv1a-GNA showed 100% mortality over 24 hours at all doses excluding 10µg, where 80% mortality was observed (Figure 4.18).

Based on these results, each 5µl injection contained a total of either 25µg or 50µg of recombinant ωACTXHv1a-Avidin fusion protein (quantified by SDS-PAGE) re-suspended in PBS. At those amounts, if the fusion protein activity was comparable to ωACTXHv1a-GNA, or if the toxin component of the fusion protein was active, mortality should be observed. Injections of PBS were also carried out as a negative control.

The recombinant ωACTXHv1a-Avidin fusion protein was found to have no insecticidal activity in *M. brassicae* larvae (Figure 4.18). Injected larvae were completely unaffected by the fusion protein, with 100% survival and no obvious visual or developmental effects being observed. Similar survival was observed in the PBS negative control injections (Figure 4.18). The absence of mortality at a 50µg dose of recombinant ωACTXHv1a-Avidin suggests that the ωACTXHv1a toxin used in this fusion protein is also inactive, presumably as a result of incorrect folding.

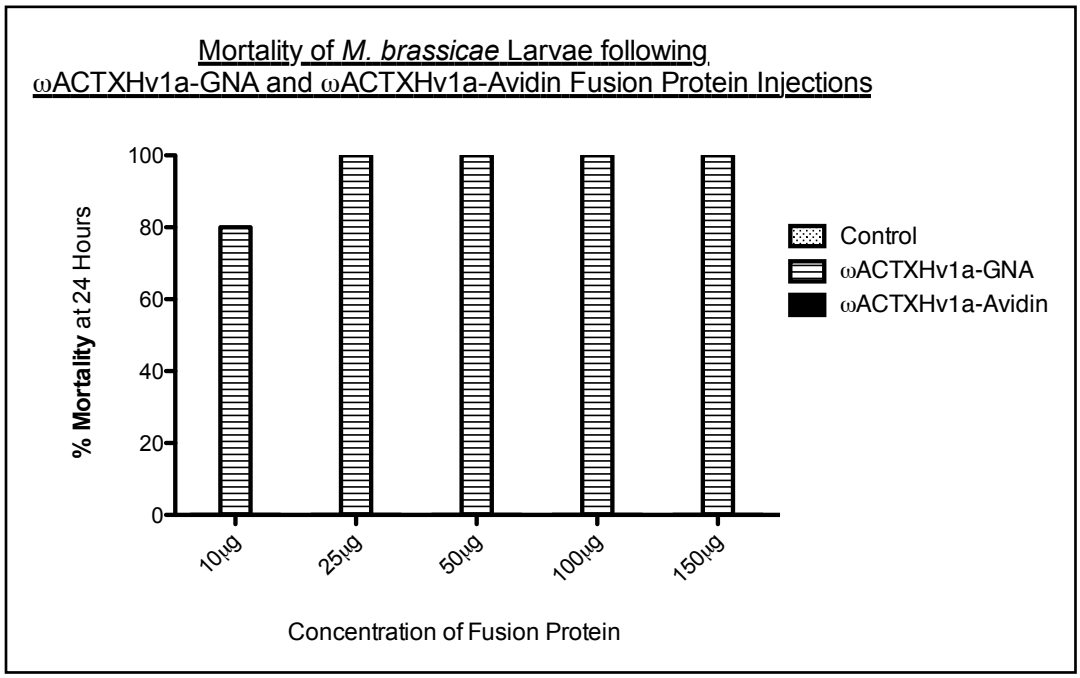

Figure 4.18.

Mortality of *M. brassicae* larvae following injection with varying amounts of recombinant ωACTXHv1a -GNA or ωACTXHv1a-Avidin fusion protein.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

eGFP-Avidin (enhanced Green Fluorescent Protein) Fusion Protein

To establish if the functionality of a protein is retained following fusion to avidin, a non-toxin alternative was used.

1. Production of the eGFP-Avidin Fusion Protein Expression Construct

An expression construct for a fusion protein incorporating enhanced Green Fluorescent Protein (eGFP) as a replacement for a toxin was produced using the cloning strategy described in Chapter 2. The eGFP-Avidin construct contained eGFP fused N-terminally to avidin using a *Not*I restriction site linker (amino acid sequence: AAAA). The complete fusion protein expression construct was cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequence, deduced amino acid sequence and schematic diagram of the expression construct is shown in Figure 4.19.

```
ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA    +75
 M  R  F  P  S  I  F  T  A  V  L  F  A  A  S  S  A  L  A  A  P  V  N  T  T      25

ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT    +150
 T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G  Y  S  D  L  E  G  D  F  D  V      50

GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT    +225
 A  V  L  P  F  S  N  S  T  N  N  G  L  L  F  I  N  T  T  I  A  S  I  A  A      75

AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCAATGGTGAGCAAGGGCGAGGAGCTGTTC    +300
 K  E  E  G  V  S  L  E  K  R  E  A  E  A  A  A  M  V  S  K  G  E  E  L  F      100

ACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC    +375
 T  G  V  V  P  I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G      125

GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCC    +450
 E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P      150

ACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTC    +525
 T  L  V  T  T  L  T  Y  G  V  Q  C  F  S  R  Y  P  D  H  M  K  Q  H  D  F      175

TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACC    +600
 F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T      200

CGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGAC    +675
 R  A  E  V  K  F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D      225

GGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAG    +750
 G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M  A  D  K  Q  K      250

AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAG    +825
 N  G  I  K  V  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A  D  H  Y  Q      275

CAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGC    +900
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GACGAGCTGTACAAGGCGGCCGCCGCTAGAAAATGCTCGCTGACTGGGAAATGGACCAACGATCTGGGCTCCAAC    +1050
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ATGACCATCGGGGCTGTGAACAGCAAAGGTGAATTCACAGGCACCTACACCACAGCCGTAACAGCCACATCAAAT    +1125
 M  T  I  G  A  V  N  S  K  G  E  F  T  G  T  Y  T  T  A  V  T  A  T  S  N      375

GAGATCAAAGAGTCACCACTGCATGGGACACAAAACACCATCAACAAGAGGACCCAGCCCACCTTTGGCTTCACC    +1200
 E  I  K  E  S  P  L  H  G  T  Q  N  T  I  N  K  R  T  Q  P  T  F  G  F  T      400

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CTGAAGACCATGTGGCTGCTGCGGTCAAGTGTTAATGACATTGGTGATGACTGGAAAGCTACGCGTGTTGGTATC    +1350
 L  K  T  M  W  L  L  R  S  S  V  N  D  I  G  D  D  W  K  A  T  R  V  G  I      450

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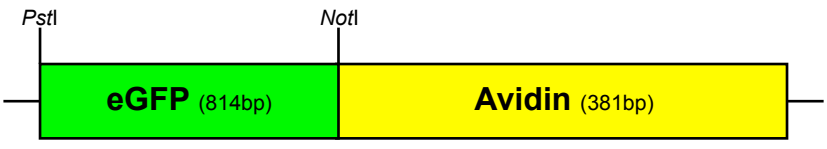

Figure 4.19.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the eGFP-Avidin fusion protein - 'eGFP (AAAA) Avidin.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The eGFP sequence is highlighted in green. The avidin sequence is highlighted in yellow. Diagram not to scale.

2. Recombinant Expression of the eGFP-Avidin Fusion Protein

The fusion protein expression construct vector was transformed into protease-deficient *P. pastoris*. Clones expressing the fusion protein were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. A high expresser was cultured in a bench-top fermenter.

Recombinant eGFP-Avidin was partially purified from culture supernatant by phenyl-Sepharose hydrophobic interaction chromatography. A typical elution profile from a phenyl-Sepharose column chromatography purification is shown in Figure 4.20.

From the amino acid sequence, the predicted size of eGFP-Avidin is 41.4kDa. The presence of eGFP-Avidin was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluate was also analysed by SDS-PAGE (Figure 4.21).

Recombinant eGFP-Avidin was present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequence (46kDa) (Figure 4.21(b)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The SDS-PAGE analysis showed the presence of some high molecular weight proteins (>66kDa) (Figure 4.21(a)).

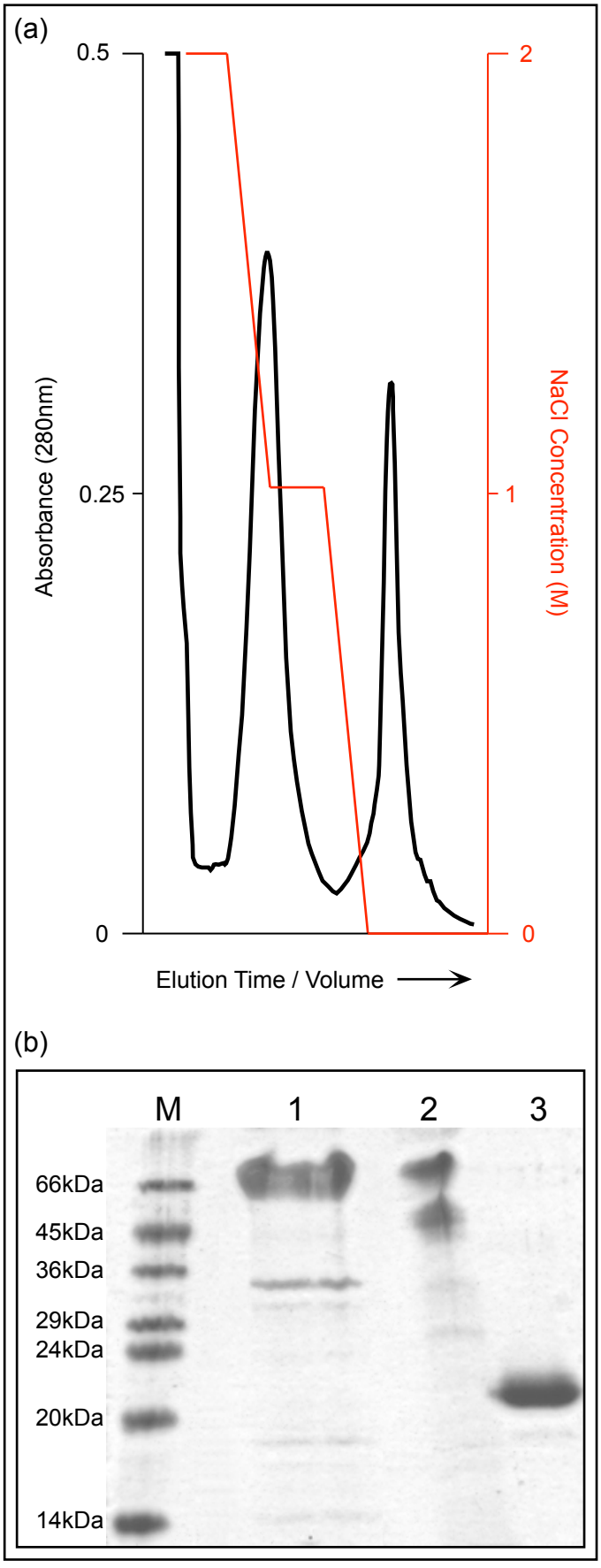

Figure 4.20.

Phenyl-Sepharose column purification.
(a) Typical purification profile. As indicated by the red NaCl concentration line, the NaCl gradient was held at 1M to allow elution of protein through the complete column volume. Typically, the 1M NaCl elution peak contained the majority of the larger molecular weight proteins associated with *P. pastoris* yeast. Fusion proteins were present in the 0M NaCl (water) elution peak.
(b) Typical 15% SDS-PAGE analysis of a phenyl-Sepharose column purification. M is SDS7 molecular weight marker. Lane 1 is 1M NaCl elution peak. Lane 2 is 0M NaCl (water) elution peak. (25µl loaded for each sample). Lane 3 is 5µg of recombinant avidin (20kDa). (In this case, the fusion protein was eGFP-Avidin).

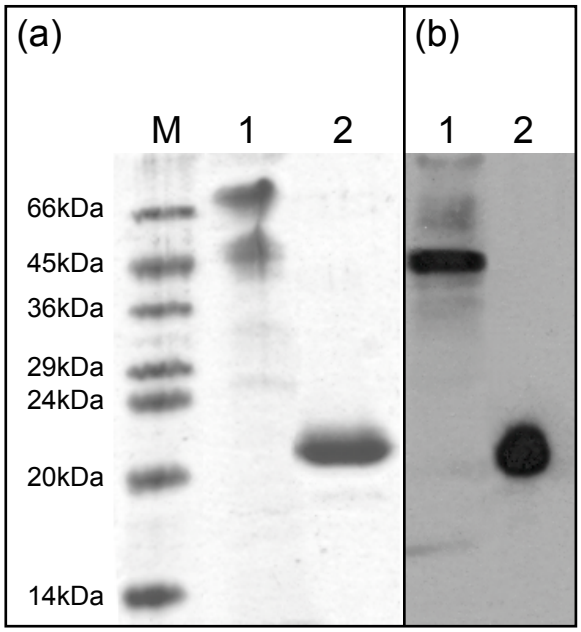

Figure 4.21.

SDS-PAGE and western analysis of the recombinant eGFP-Avidin fusion protein produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) 15% SDS-PAGE analysis. Lane 1 is 25µl of phenyl-Sepharose column elution, showing eGFP-Avidin (46kDa) with high molecular weight yeast proteins (>66kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (b) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified eGFP-Avidin (46kDa). Lane 2 is 10ng of recombinant avidin (20kDa).

The partially purified protein did not show fluorescence, suggesting the fusion protein was inactive. To check this was not due to the presence of other proteins, the partially purified final product seen in Figure 4.21(a) was subjected to Sephacryl S-200 gel filtration to exclude high molecular weight proteins but degraded during the process.

ButaIT-GNA Fragment-Avidin (ButaIT-Gavidin) Fusion Proteins

The amino acid sequences of the ButaIT-Avidin 2 fusion protein produced earlier (page 96), and of the ButaIT-GNA fusion protein produced by Pham-Trung *et al.* (2006), were analysed for structural features using Protean (DNASTAR Lasergene 9 suite). These analyses revealed that the avidin fusion protein has regions of positive charge on either side of the fusion site, a major difference when compared to the GNA fusion protein, which has a region of negative charge at the N-terminal end of GNA (Figure 4.22).

1. Production of the ButaIT-GNA Fragment-Avidin Fusion Protein Expression Construct

Based on the charge difference highlighted in Figure 4.22, a ButaIT-Avidin fusion protein expression construct incorporating a fragment of GNA as a linker (designated ButaIT-Gavidin) was produced using the cloning strategy described in Chapter 2. The GNA fragment was incorporated to introduce a negatively charged region between the ButaIT toxin and avidin (Figure 4.23). The ButaIT-Gavidin construct contained ButaIT toxin fused N-terminally to avidin using the first 17 amino acids of GNA as a linker (amino acid sequence: DNILYSGETLSTGEFLN). The expression construct also had a C-terminal extension encoding a $(His)_6$ tag. The complete fusion protein expression construct was cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequence, deduced amino acid sequence and schematic diagram of the expression construct is shown in Figure 4.24.

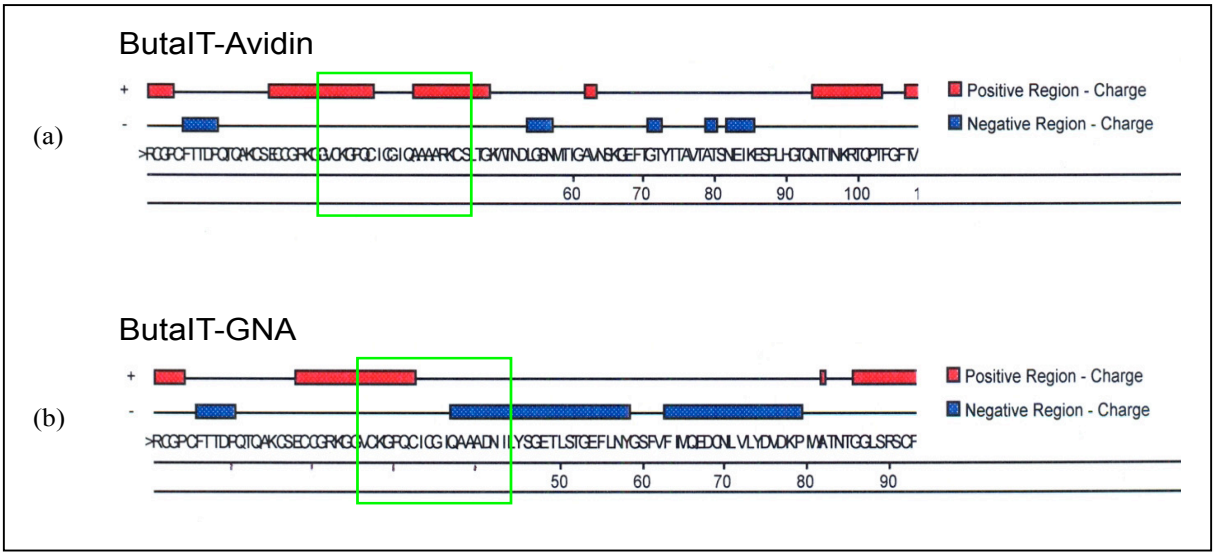

Figure 4.22.

Fusion protein secondary structure charge analysis using Protean (DNASTAR Lasergene 9 suite).
(a) ButaIT-Avidin. (b) ButaIT-GNA.
The green boxes outline the areas of charge difference between the two fusion proteins.

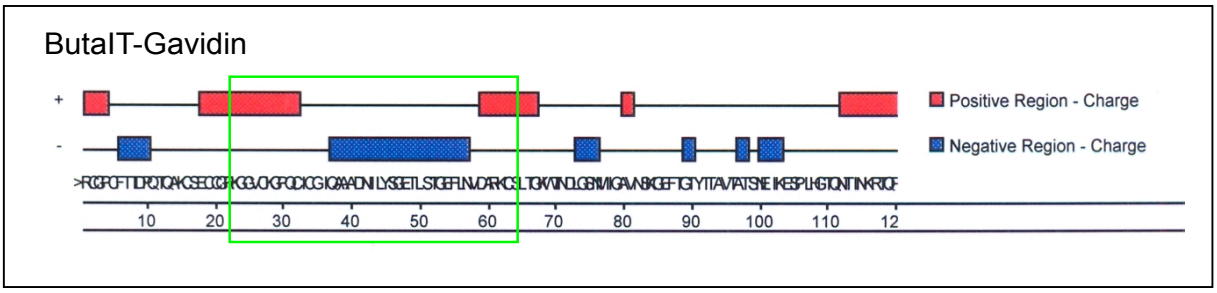

Figure 4.23.

ButaIT-Gavidin fusion protein secondary structure charge analysis using Protean (DNASTAR Lasergene 9 suite).
The green box outlines the newly added negative charge (GNA fragment) between the ButaIT toxin and avidin.

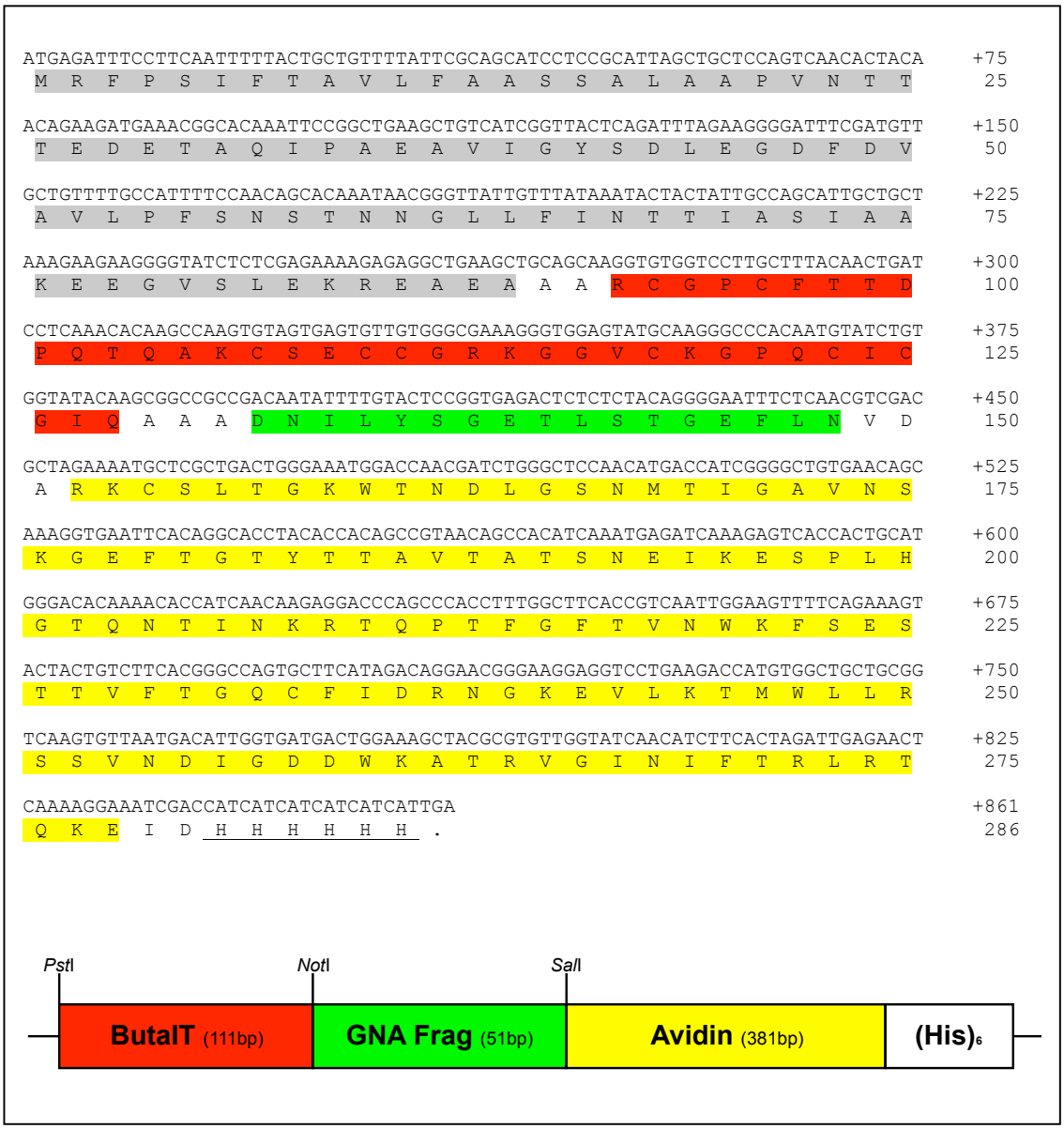

Figure 4.24.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ButaIT-Gavidin fusion protein - 'ButaIT (GNA Fragment) Avidin $(His)_6$.'

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted red. The GNA fragment sequence is highlighted in green. The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black. Diagram not to scale.

2. Recombinant Expression of the ButaIT-Gavidin Fusion Protein

The fusion protein expression construct vector was transformed into protease-deficient *P. pastoris*. Clones expressing the fusion protein were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. A high expresser was cultured in a bench-top fermenter.

Recombinant ButaIT-Gavidin was purified from culture supernatant by nickel affinity chromatography utilising the added $(His)_6$ tag (Figure 4.4).

From the amino acid sequence, the predicted size of ButaIT-Gavidin is 21.5kDa. The presence of ButaIT-Gavidin was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluate was also analysed by SDS-PAGE (Figure 4.25).

Recombinant ButaIT-Gavidin was present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequence (26kDa) (Figure 4.25(b)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The SDS-PAGE analysis showed that the purified ButaIT-Gavidin fusion protein comprised less than 50% of the protein isolated, with three degradation fragments also observed (21kDa, 20.5kDa and 20kDa) (Figure 4.25(a)).

Once analysed, column eluate was dialysed to remove salts and lyophilised by freeze-drying. Lyophilised protein was quantified by SDS-PAGE (Figure 4.7). Due to relatively low solubility, a yield of approximately 2mg of fusion protein per litre of culture supernatant was obtained.

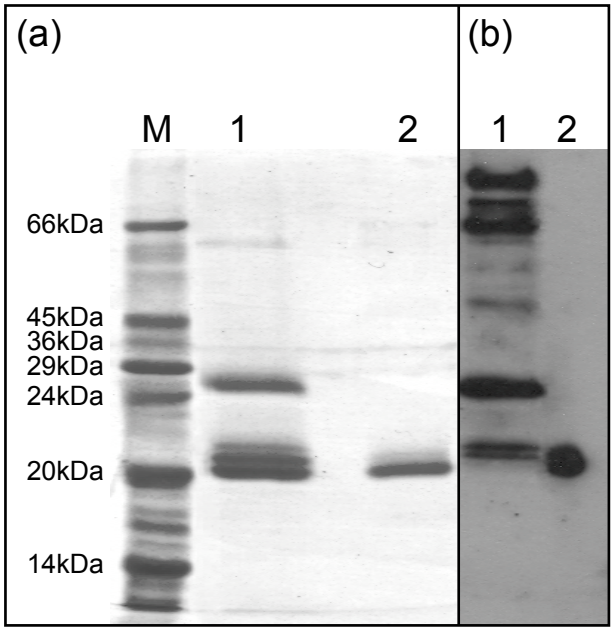

Figure 4.25.

SDS-PAGE and western analysis of the recombinant ButaIT-Gavidin fusion protein produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) 15% SDS-PAGE analysis. Lane 1 is 20µl of nickel column elution, showing ButaIT-Gavidin (26kDa) with proteolytic degradation products (21kDa, 20.5kDa and 20kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (b) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 4µl of fermenter supernatant, showing unpurified ButaIT-Gavidin (26kDa) with high molecular weight yeast proteins and proteolytic degradation products (21kDa and 20.5kDa). Lane 2 is 10ng of recombinant avidin (20kDa).

3. Insecticidal Activity of the Recombinant ButaIT-Gavidin Fusion Protein

The recombinant ButaIT-Gavidin fusion protein was initially tested for insecticidal activity by injection into newly moulted fifth stadium *M. brassicae* larvae. Each 5µl injection contained a total of 50µg of fusion protein (quantified by SDS-PAGE) re-suspended in PBS.

All of the injected *M. brassicae* larvae were paralysed immediately and did not recover, showing an almost instant death, suggesting that an active fusion protein with avidin had finally been produced.

Following these preliminary injections, a full evaluation of the insecticidal activity of recombinant ButaIT-Gavidin was carried out by injecting amounts in the

range 1μg-50μg into newly moulted fifth stadium *M. brassicae* larvae. Recombinant ButaIT-GNA was injected as a comparison and PBS was used as a negative control.

Recombinant ButaIT-Gavidin was effective (causing mortality) at doses as low as 2.5μg, whereas recombinant ButaIT-GNA was only effective from a dose of 20μg (Figure 4.26). Complete mortality was observed following injection with 10μg-50μg of recombinant ButaIT-Gavidin and the instant toxicity described above was observed in all cases. Similar instant effects were observed in the lower doses (1μg-10μg) in which mortality was recorded. The larvae that survived the initial injection progressed normally to pupation. A survival level of 100% was observed in the PBS negative control injections (Figure 4.26).

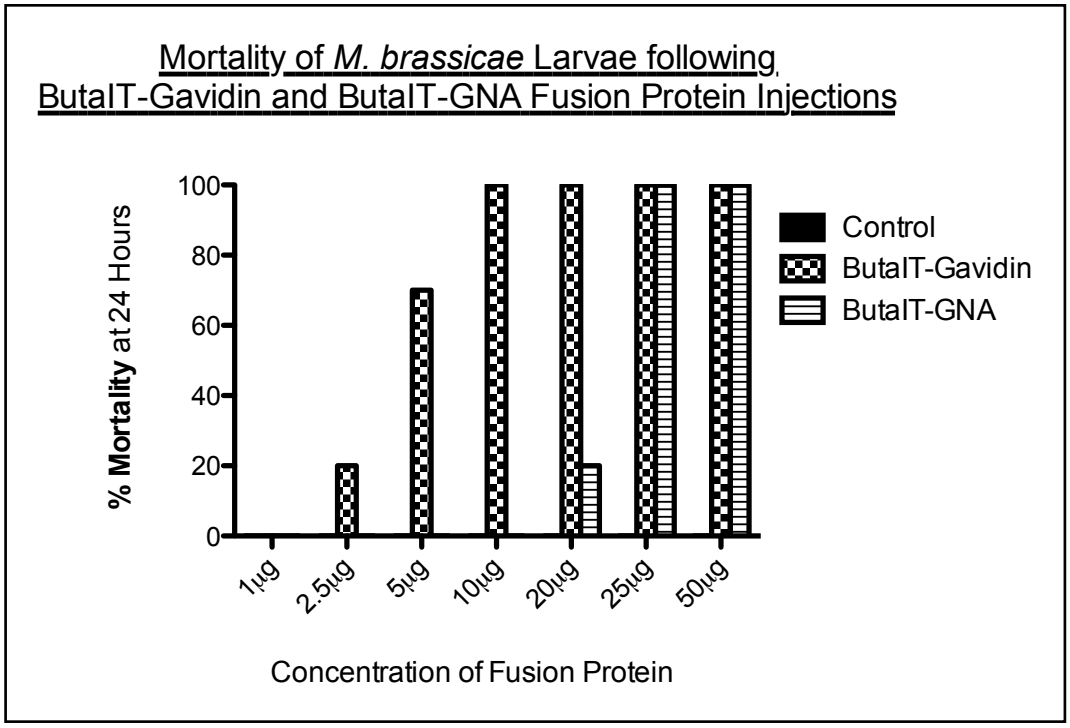

Figure 4.26.

Mortality of *M. brassicae* larvae following injection with varying amounts of recombinant ButaIT-Gavidin or ButaIT-GNA fusion protein.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

The oral activity of recombinant ButaIT-Gavidin towards *M. brassicae* larvae was also evaluated by feeding droplets containing total amounts of recombinant

ButaIT-Gavidin in the range 0.5μg-50μg. Droplets of PBS were fed to larvae as a negative control for the bioassay.

Recombinant ButaIT-Gavidin was effective (causing mortality) at doses as low as 1μg, with 100% mortality observed in all cases excluding the 0.5μg and 1μg doses (Figure 4.27). A survival level of 100% was observed in the PBS droplet fed negative control larvae (Figure 4.27). The results suggest that recombinant ButaIT-Gavidin is as effective via oral delivery as it is through injection. In contrast to the instant effects observed following injection, droplet fed *M. brassicae* larvae showed paralysis of the hind abdominal and anal prolegs for approximately 20 minutes before their eventual mortality. The larvae continued to move around either by dragging their paralysed abdominal region or lifting it and holding it clear of the bench surface.

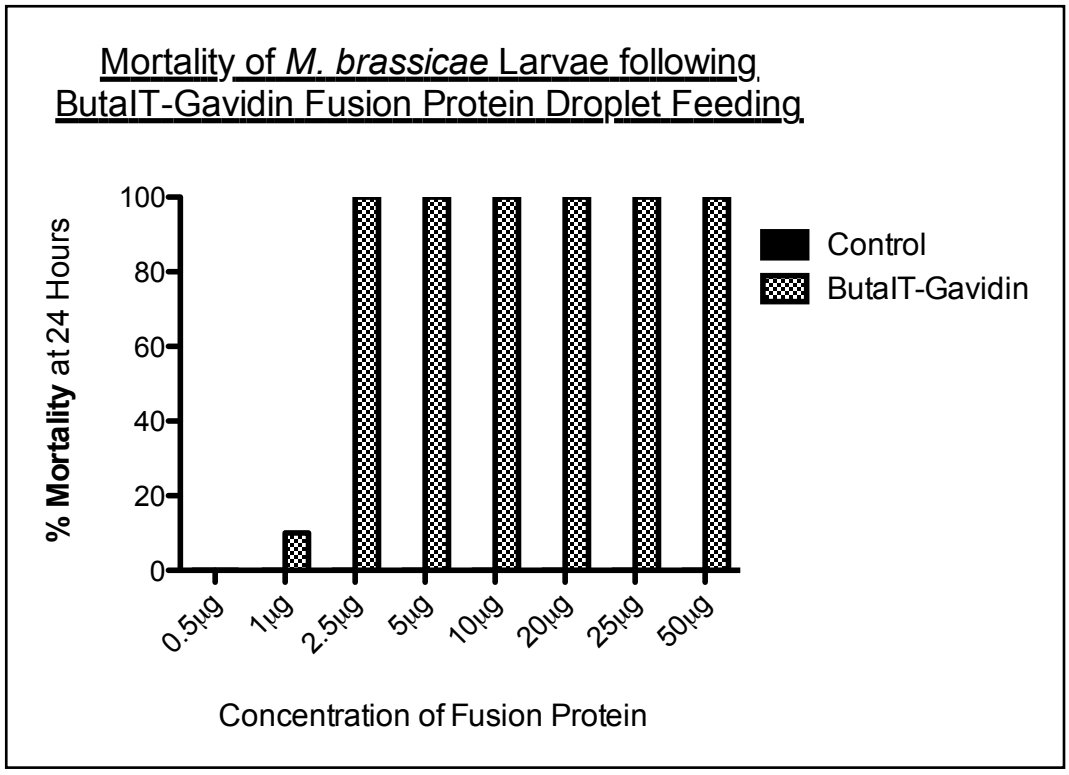

Figure 4.27.

Mortality of *M. brassicae* larvae fed a droplet containing varying amounts of recombinant ButaIT-Gavidin fusion protein.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

4. N-Terminal Sequencing of the Recombinant ButaIT-Gavidin Fusion Protein Fragments

As the recombinant ButaIT-Gavidin fusion protein showed insecticidal activity, an investigation into how to improve the production yield was conducted. As seen in Figure 4.25, the intact fusion protein only accounted for approximately 40% of the total protein content, with three degradation fragments making up the remaining 60%. A western blot of recombinant ButaIT-Gavidin was carried out using anti-avidin, anti-GNA and anti-His-tag antibodies to establish which of the fragments reacted with each specific antibody (Figure 4.28).

The results suggest that avidin was present within all of the degradation fragments, albeit to a lesser extent in the smallest (Figure 4.28(a)). Only the largest degradation fragment contained enough GNA to react with the GNA antibody (Figure 4.28(b)). All three degradation fragments contained the $(His)_6$ tag (Figure 4.28(c)).

From the nucleotide sequence of the ButaIT-Gavidin construct (Figure 4.24), it was speculated that the cleavage of the fusion protein occurred within the GNA fragment. However, to identify the exact position of the cleavage sites, protein N-terminal sequencing was conducted by Cambridge Peptides (Philip Victor Road, Birmingham, West Midlands, UK).

The protein N-terminal sequencing (Table 4.2) confirmed that the degradation of the recombinant ButaIT-Gavidin fusion protein was a result of the action of one or more chymotrypsin-like protease at two specific cleavage sites within the GNA fragment. The major product resulted from cleavage after amino acid residue 10 (L) in the GNA fragment, with a less abundant product resulting from cleavage after amino acid residue 16 (L). A third potential chymotrypsin site is also present in the GNA fragment region after amino acid residue 4 (L) (Figure 4.29).

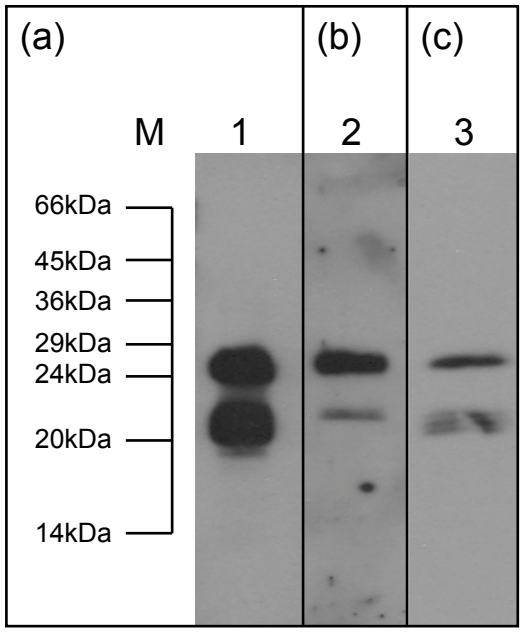

Figure 4.28.

Western analysis of the recombinant ButaIT-Gavidin fusion protein degradation fragments.

One second exposures of the membrane transferred from a 17.5% SDS-PAGE gel. M is SDS7 molecular weight marker. (a) Anti-avidin antibody (1:10000). Lane 1 is 25ng of ButaIT-Gavidin, showing ButaIT-Gavidin (26kDa) and three proteolytic degradation fragments (21kDa, 20.5kDa and 20kDa). (b) Anti-GNA antibody (1:3000). Lane 2 is 50ng of ButaIT-Gavidin, showing ButaIT-Gavidin (26kDa) and one proteolytic degradation fragment (21kDa). (c) Anti-His tag antibody (1:1000). Lane 3 is 200ng of ButaIT-Gavidin, showing ButaIT-Gavidin (26kDa) and three proteolytic degradation fragments (21kDa, 20.5kDa and 20kDa).

Residue Number	Read 1	Read 2	Read 3
1	S	N	A
2	T	E	V
3	G	D	F
4	E	A	L
5	F	N	R

Table 4.2.

N-terminal sequence analysis of the ButaIT-Gavidin fusion protein degradation fragments.

Read 1 identifies the STGEF sequence at the N-terminus of a ButaIT-Gavidin fragment. Reads 2 and 3 identify the NVDAR sequence at the N-terminus of another fragment.

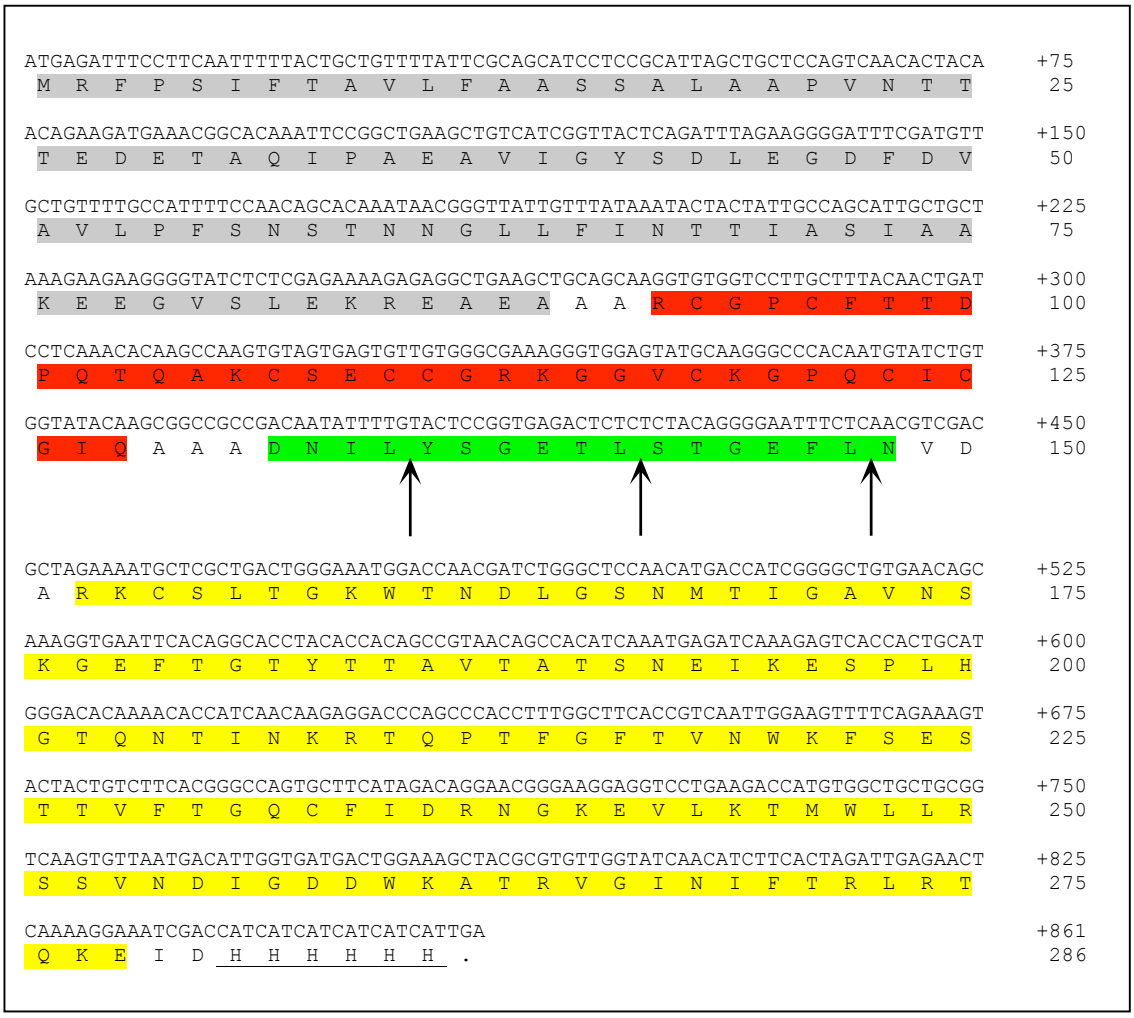

Figure 4.29.

Nucleotide sequence and deduced amino acid sequence of the ButaIT-Gavidin fusion protein showing chymotrypsin cleavage sites within the GNA fragment.

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted red. The GNA fragment sequence is highlighted in green. The arrows indicate chymotrypsin cleavage sites. The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black.

5. Production of Mutated ButaIT-Gavidin Fusion Protein Expression Constructs

Based on the protein N-terminal sequencing results, two mutated variants of ButaIT-Gavidin were produced to reduce the presence of the cleavage fragments. One fusion protein, designated ButaIT-Gavidin 1 Mutation, contained one mutated chymotrypsin enzyme site, while the other fusion protein, designated ButaIT-Gavidin 3 Mutations, included three mutated chymotrypsin enzyme sites. The mutations were simple nucleotide base alterations introduced by site-directed mutagenesis as described in Chapter 2. The nucleotide and deduced amino acid sequences of the expression constructs are shown in Figures 4.30 and 4.31.

From Figure 4.30, the ButaIT-Gavidin 1 Mutation has 421C>G and 422T>C nucleotide base changes, creating a L141A amino acid change. From Figure 4.31, the ButaIT-Gavidin 3 Mutations has 403T>G, 404T>C, 406T>A, 407A>C, 421C>G, 422T>C, 439C>G and 440T>C nucleotide base changes, creating L135A, Y136T, L141A and L147A amino acid changes.

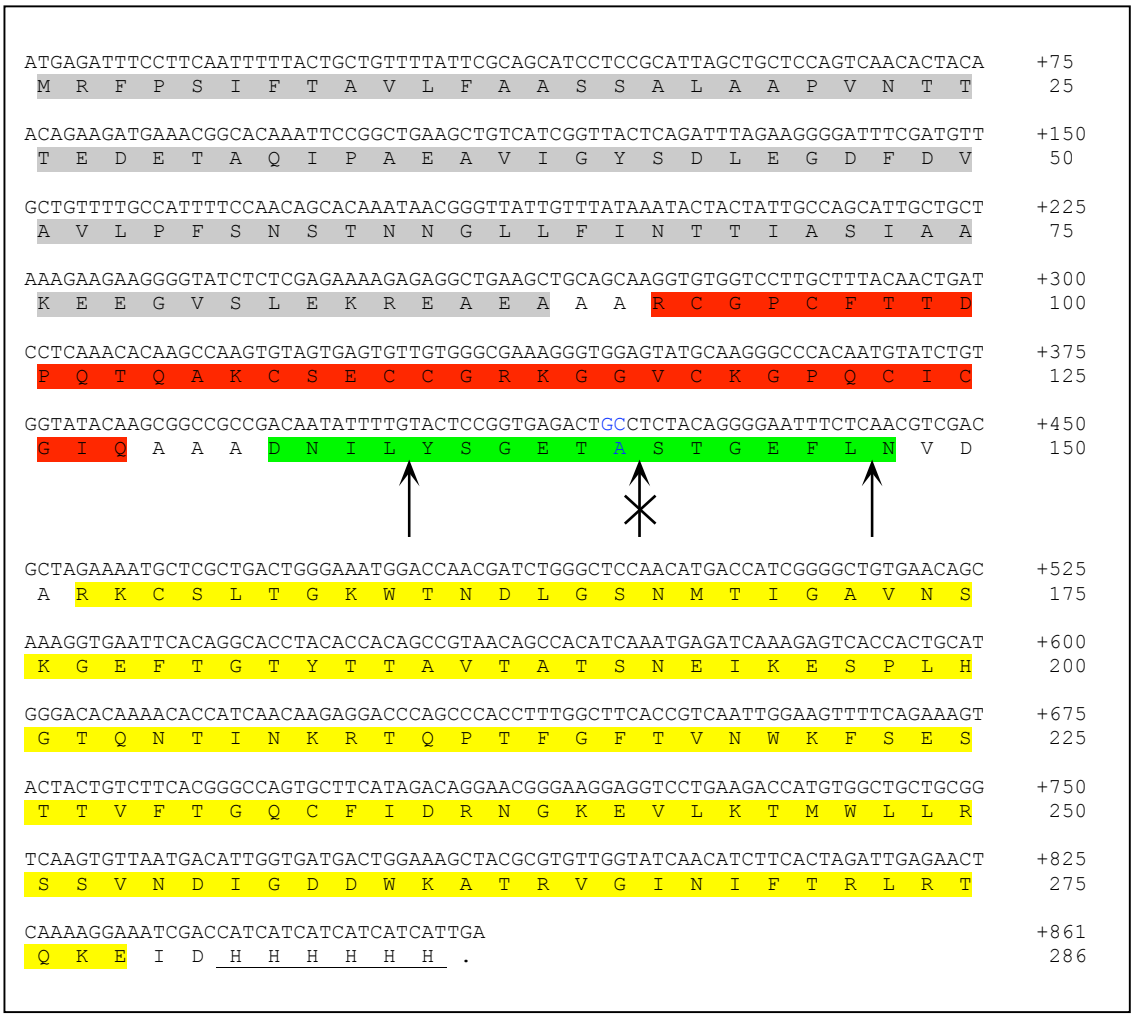

Figure 4.30.

Nucleotide sequence and deduced amino acid sequence of the ButaIT-Gavidin 1 Mutation fusion protein showing the mutated chymotrypsin cleavage site within the GNA fragment.
(421C>G and 422T>C nucleotide base change; L141A amino acid change).

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted red. The GNA fragment sequence is highlighted in green. The arrows indicate chymotrypsin cleavage sites. The crossed arrow indicates the mutation site (sequence changes coloured blue). The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black.

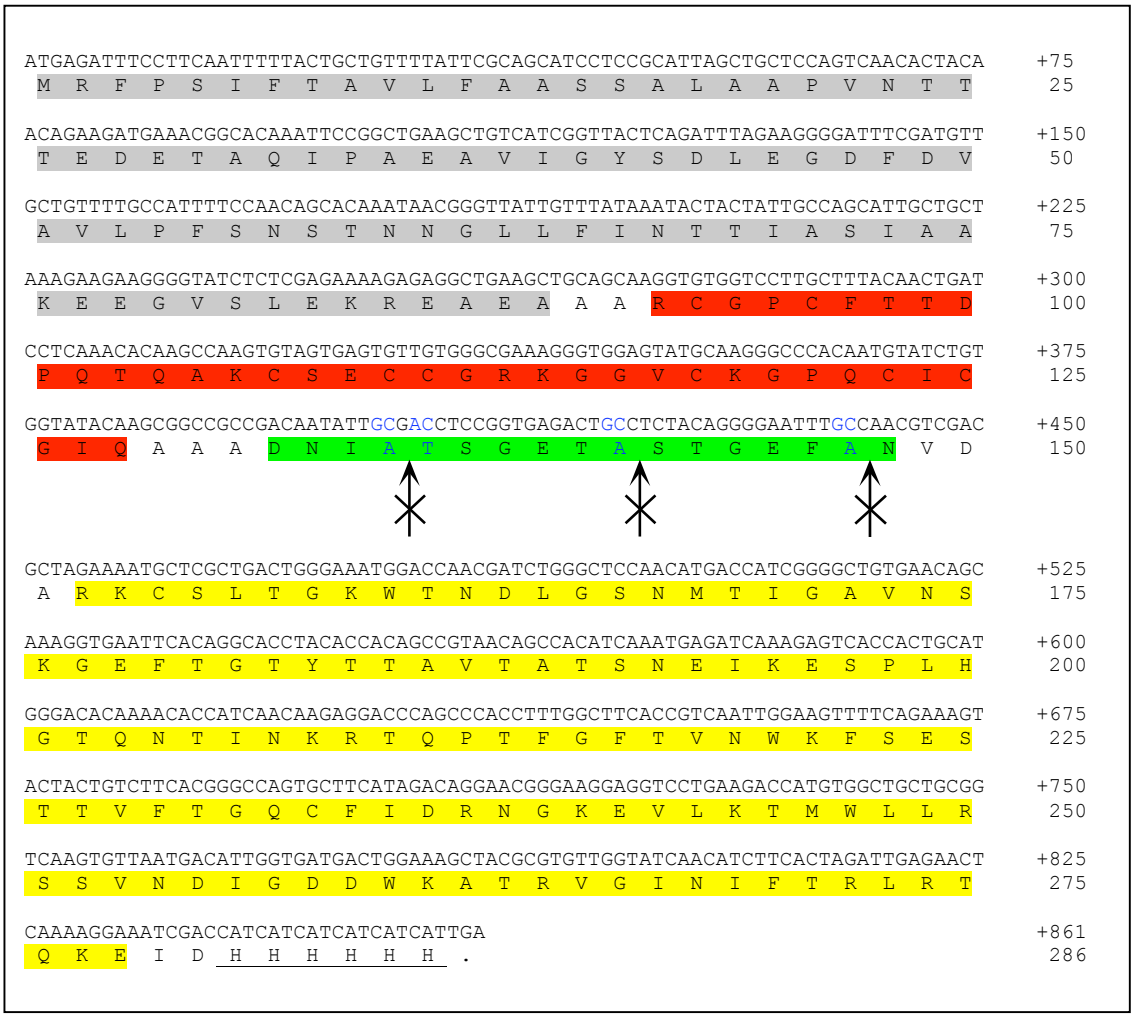

Figure 4.31.

Nucleotide sequence and deduced amino acid sequence of the ButaIT-Gavidin 3 Mutations fusion protein showing the mutated chymotrypsin cleavage sites within the GNA fragment.
(403T>G, 404T>C, 406T>A, 407A>C, 421C>G, 422T>C, 439C>G and 440T>C nucleotide base changes; L135A, Y136T, L141A and L147A amino acid changes).

The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted red. The GNA fragment sequence is highlighted in green. The crossed arrows indicate the chymotrypsin cleavage mutation sites (sequence changes coloured blue). The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black.

6. Recombinant Expression of the Mutated ButaIT-Gavidin Fusion Proteins

The mutated ButaIT-Gavidin fusion protein expression construct vectors were transformed into protease-deficient *P. pastoris*. Clones expressing the fusion proteins were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. High expressers were cultured separately in bench-top fermenters.

Both recombinant ButaIT-Gavidin 1 Mutation and ButaIT-Gavidin 3 Mutations were purified from culture supernatant by nickel affinity chromatography utilising the added $(His)_6$ tag (Figure 4.4).

From the amino acid sequences, the predicted sizes of ButaIT-Gavidin 1 Mutation and ButaIT-Gavidin 3 Mutations are 21.4kDa and 21.3kDa respectively. The presence of the mutated ButaIT-Gavidin fusion proteins was confirmed by western blotting (anti-avidin antibodies) of culture supernatant from the fermentation, and column eluates were also analysed by SDS-PAGE (Figure 4.32).

Recombinant ButaIT-Gavidin 1 Mutation and ButaIT-Gavidin 3 Mutations were present and reacted with anti-avidin antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequences (24kDa and 24.5kDa respectively) (Figure 4.32(a)(ii) and (b)(ii)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The purified ButaIT-Gavidin 1 Mutation shows only two cleavage fragments (21kDa and 20.5kDa) (Figure 4.32(a)), an improvement over the original ButaIT-Gavidin where three were observed (21kDa, 20.5kDa and 20kDa) (Figure 4.25(a)). Although the purified ButaIT-Gavidin 3 Mutations shows a marked reduction in the three original cleavage fragments (21kDa, 20.5kDa and 20kDa), two completely new cleavage fragments were observed (23kDa and 22kDa) (Figure 4.32(b)).

Once analysed, column eluates were dialysed to remove salts and lyophilised by freeze-drying. Lyophilised proteins were quantified by SDS-PAGE (Figure 4.7). Yields of approximately 10mg of ButaIT-Gavidin 1 Mutation fusion protein and 20mg of ButaIT-Gavidin 3 Mutations fusion protein per litre of culture supernatant were obtained.

(a) ButaIT-Gavidin 1 Mutation (b) ButaIT-Gavidin 3 Mutations

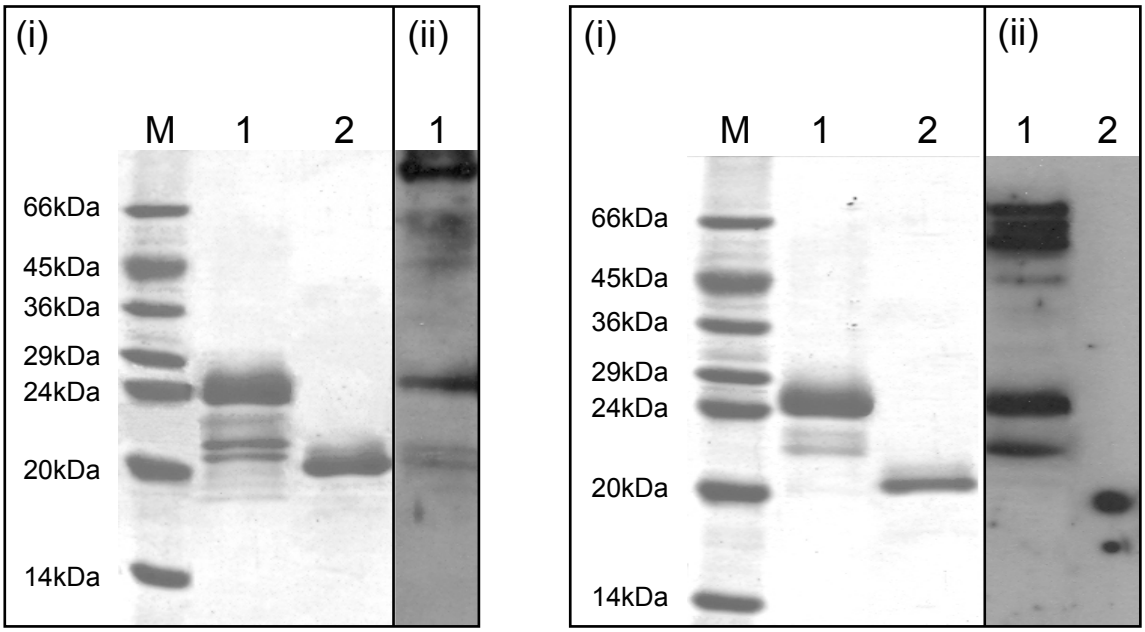

Figure 4.32.

SDS-PAGE and western analysis of the recombinant mutated ButaIT-Gavidin fusion proteins produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) ButaIT-Gavidin 1 Mutation: (i) 15% SDS-PAGE analysis. Lane 1 is 20μl of nickel column elution, showing ButaIT-Gavidin 1 Mutation (24kDa) with two cleavage fragments (21kDa and 20.5kDa). Lane 2 is 5μg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1μl of fermenter supernatant, showing unpurified ButaIT-Gavidin 1 Mutation (24kDa) with two cleavage fragments (21kDa and 20.5kDa) and high molecular weight yeast proteins. (b) ButaIT-Gavidin 3 Mutations: (i) 15% SDS-PAGE analysis. Lane 1 is 15μl of nickel column elution, showing ButaIT-Gavidin 3 Mutations (24.5kDa) with two cleavage fragments (23kDa and 22kDa). Lane 2 is 5μg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1μl of fermenter supernatant, showing unpurified ButaIT-Gavidin 3 Mutations (24.5kDa) with one cleavage fragment (22kDa) and high molecular weight yeast proteins. Lane 2 is 10ng of recombinant avidin (20kDa).

7. Insecticidal Activity of the Recombinant Mutated ButaIT-Gavidin Fusion Proteins

The recombinant mutated ButaIT-Gavidin fusion proteins were tested for insecticidal activity by injection into newly moulted fifth stadium *M. brassicae* larvae. Each 5μl injection contained a total of 50μg of fusion protein (quantified by SDS-PAGE) re-suspended in PBS. The original recombinant ButaIT-Gavidin was injected as a comparison and PBS was used as a negative control.

The recombinant ButaIT-Gavidin 1 Mutation fusion protein showed toxicity by injection (Figure 4.33). Complete mortality was observed in 48 hours and the effects were similar to the original recombinant ButaIT-Gavidin but 24 hours later. In contrast, the recombinant ButaIT-Gavidin 3 Mutations fusion protein showed no insecticidal activity (Figure 4.33). A survival level of 100% was observed in the PBS negative control injections (Figure 4.33). The activity of recombinant ButaIT-Gavidin 1 Mutation over 48 hours and non-toxicity of recombinant ButaIT-Gavidin 3 Mutations when compared to the original recombinant ButaIT-Gavidin (with activity over 24 hours) suggests that *in vivo* chymotrypsin cleavage of the ButaIT-Gavidin fusion protein is necessary for it to be active.

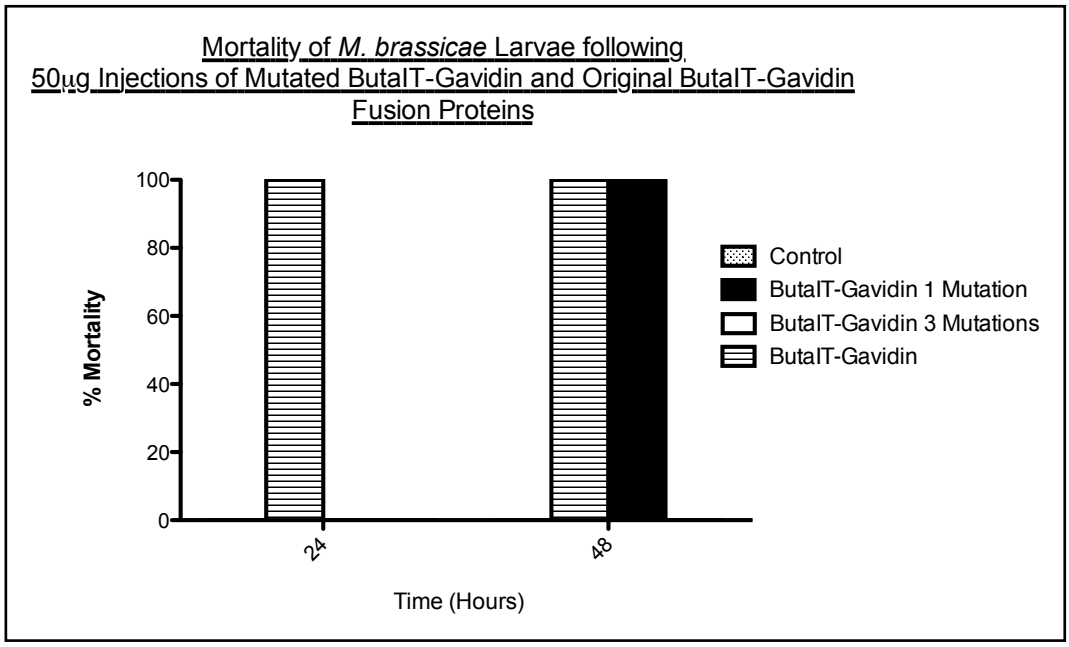

Figure 4.33.

Mortality of *M. brassicae* larvae following injection with 50µg of recombinant mutated ButaIT-Gavidin or ButaIT-Gavidin fusion proteins.

(n = 20 for each treatment - Two separate treatments with 10 individuals).

The oral activity of the recombinant mutated ButaIT-Gavidin fusion proteins was also evaluated by droplet feeding to newly moulted fifth stadium *M. brassicae* larvae. Each 5µl droplet contained a total of 50µg of the fusion protein. Equivalent droplets of the original recombinant ButaIT-Gavidin were fed as a comparison and PBS was fed to larvae as a negative control for the bioassay.

Both recombinant ButaIT-Gavidin 1 Mutation and ButaIT-Gavidin 3 Mutations were non-toxic when delivered orally (Figure 4.34). Larvae were completely unaffected by both of the mutated fusion proteins, with 100% survival and no obvious visual or developmental effects being observed. The original recombinant ButaIT-Gavidin droplet fed larvae showed 100% mortality in 24 hours and 100% survival was observed in the PBS negative control (Figure 4.34).

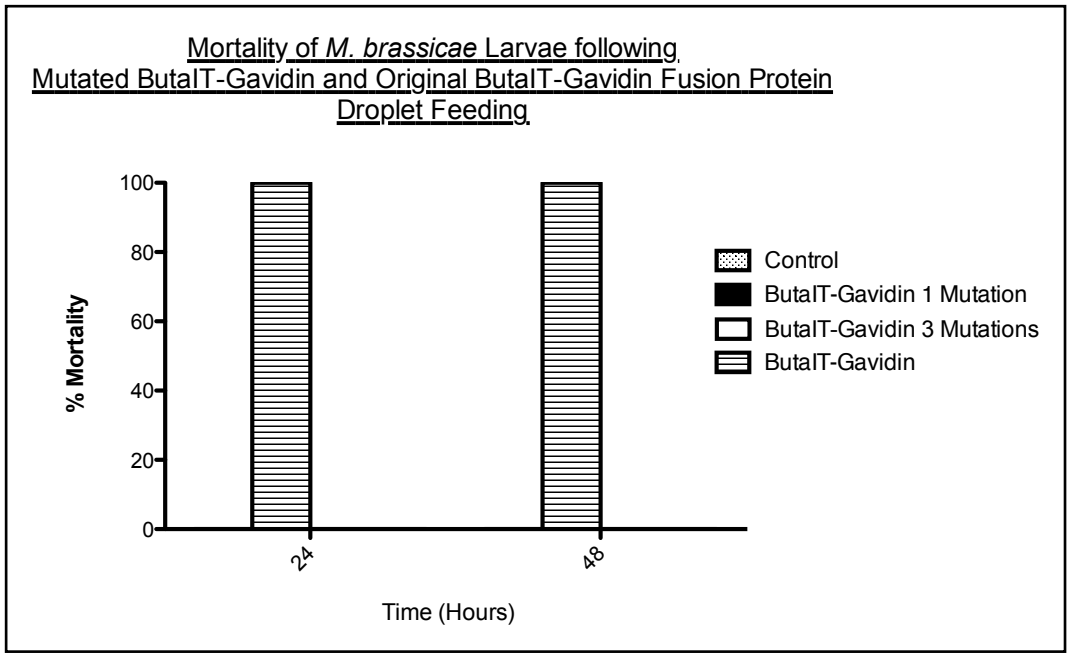

Figure 4.34.

Mortality of *M. brassicae* larvae fed a droplet containing 50µg of recombinant mutated ButaIT-Gavidin or ButaIT-Gavidin fusion proteins.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

ButaIT-GNA Fragment and GNA Fragment-Avidin Fusion Proteins

To help to identify the mode of action of ButaIT-Gavidin, more fusion proteins were produced, each containing different components of ButaIT-Gavidin.

1. Production of ButaIT-GNA Fragment and GNA Fragment-Avidin Fusion Protein Expression Constructs

Expression constructs for two fusion proteins (designated ButaIT-GNA Fragment and GNA Fragment-Avidin) were produced using the cloning strategy described in Chapter 2. The ButaIT-GNA Fragment construct contained ButaIT toxin fused N-terminally to the 17 amino acid GNA fragment (amino acid sequence: DNILYSGETLSTGEFLN) using a *Not*I restriction site linker (amino acid sequence: AAA). The expression construct also had a C-terminal extension encoding a $(His)_6$ tag. The GNA Fragment-Avidin construct contained the 17 amino acid GNA fragment (amino acid sequence: DNILYSGETLSTGEFLN) fused to Avidin using a *Sal*I restriction site linker (amino acid sequence: VDA). The expression construct also had a C-terminal extension encoding a $(His)_6$ tag. The complete expression constructs of the two fusion proteins were cloned in-frame with the yeast N-terminal α-mating factor pre-pro secretory signal within the pGAPZαB expression vector. The nucleotide sequences, deduced amino acid sequences and schematic diagrams of the expression constructs are shown in Figures 4.35 and 4.36.

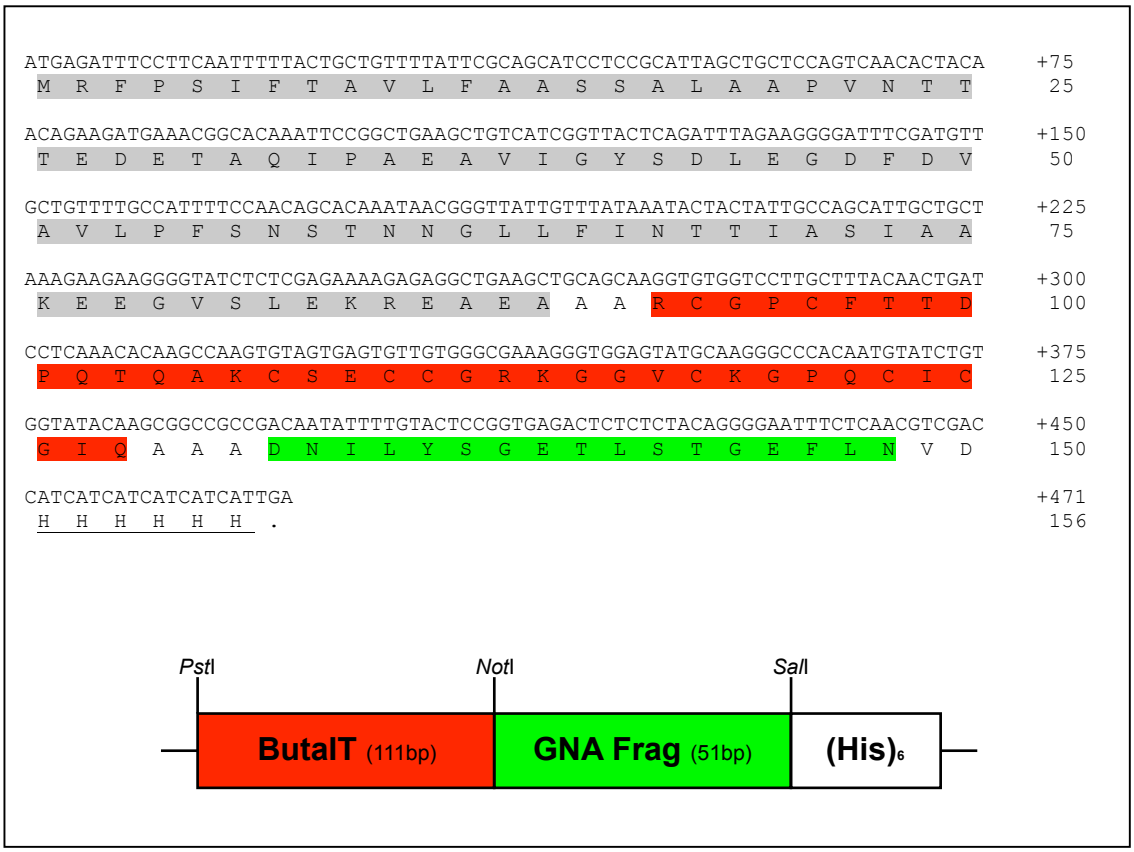

Figure 4.35.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the ButaIT-GNA Fragment fusion protein - 'ButaIT (AAA) GNA Fragment $(His)_6$.'
The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The ButaIT toxin sequence is highlighted in red. The GNA fragment sequence is highlighted in green. The $(His)_6$ tag is underlined in black. Diagram not to scale.

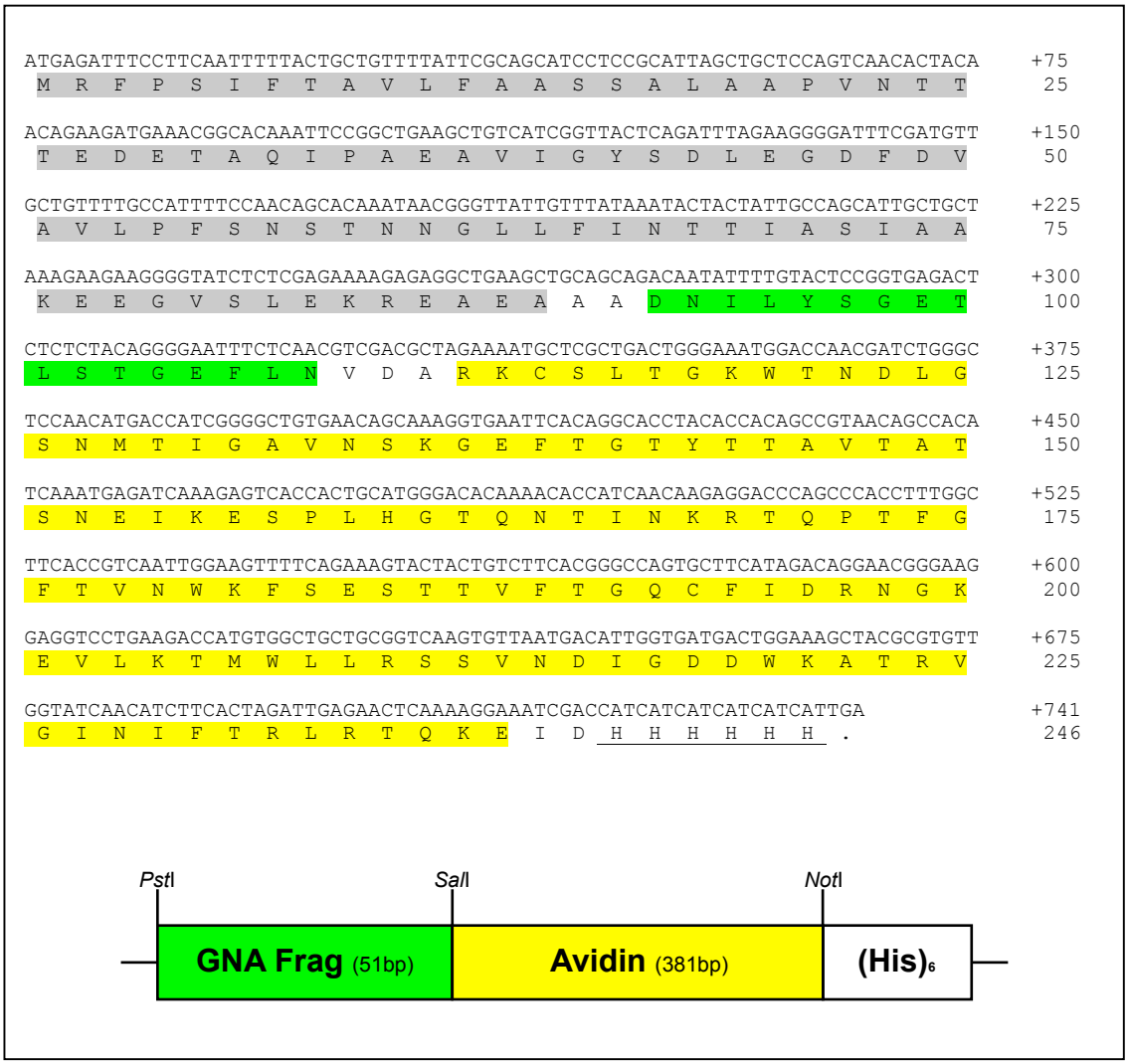

Figure 4.36.

Nucleotide sequence, deduced amino acid sequence and schematic representation of the GNA Fragment-Avidin fusion protein - 'GNA Fragment (VDA) Avidin $(His)_6$.'
The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The GNA fragment sequence is highlighted in green. The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black. Diagram not to scale.

2. Recombinant Expression of the ButaIT-GNA Fragment and GNA Fragment-Avidin Fusion Proteins

The fusion protein expression construct vectors were transformed into protease-deficient *P. pastoris*. Clones expressing the fusion proteins were identified by western blotting analysis (anti-avidin antibodies) of culture supernatant from 10ml cultures. High expressers were cultured separately in bench-top fermenters.

Both recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin were purified from culture supernatant by nickel affinity chromatography utilising the added $(His)_6$ tag (Figure 4.4).

From the amino acid sequences, the predicted sizes of ButaIT-GNA Fragment and GNA Fragment-Avidin are 7kDa and 17.4kDa respectively. The presence of ButaIT-GNA Fragment and GNA Fragment-Avidin were confirmed by western blotting (anti-GNA and anti-avidin antibodies respectively) of culture supernatant from the fermentation, and column eluates were also analysed by SDS-PAGE (Figure 4.37).

Recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin were present and reacted with the antibodies, but ran at an indicated molecular weight slightly larger than that predicted from the nucleotide sequences (10kDa and 21kDa respectively) (Figure 4.37(a)(ii) and (b)(ii)). This was most likely due to glycosylation by the *P. pastoris* host (see Chapter 3). The SDS-PAGE analysis showed a large amount of cleavage of the purified GNA Fragment-Avidin fusion protein (20.5kDa and 20kDa) (Figure 4.37(b)(i)).

Once analysed, column eluates were dialysed to remove salts and lyophilised by freeze-drying. Lyophilised proteins were quantified by SDS-PAGE (Figure 4.7). Yields of approximately 10mg of fusion protein per litre of culture supernatant were obtained for both recombinant fusion proteins.

(a) ButaIT-GNA Fragment (b) GNA Fragment-Avidin

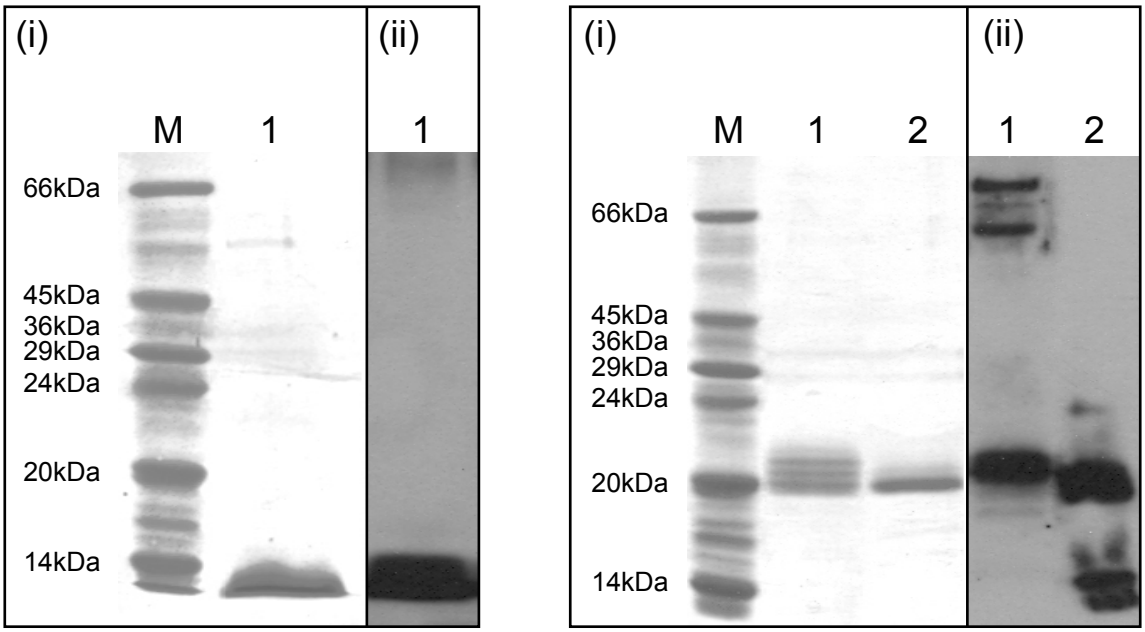

Figure 4.37.

SDS-PAGE and western analysis of the recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin fusion proteins produced in *P. pastoris*.

M is SDS7 molecular weight marker. (a) ButaIT-GNA Fragment: (i) 20% SDS-PAGE analysis. Lane 1 is 15µl of nickel column elution, showing ButaIT-GNA Fragment (10kDa). (ii) Western analysis (anti-GNA antibodies [1:3000], five second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified ButaIT-GNA Fragment (10kDa). (b) GNA Fragment-Avidin: (i) 15% SDS-PAGE analysis. Lane 1 is 15µl of nickel column elution, showing GNA Fragment-Avidin (21kDa) with proteolytic degradation products (20.5kDa and 20kDa). Lane 2 is 5µg of recombinant avidin (20kDa). (ii) Western analysis (anti-avidin antibodies [1:10000], one second exposure). Lane 1 is 1µl of fermenter supernatant, showing unpurified GNA Fragment-Avidin (21kDa) with high molecular weight yeast proteins. Lane 2 is 10ng of recombinant avidin (20kDa).

3. Insecticidal Activity of the Recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin Fusion Proteins

The recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin fusion proteins were tested for insecticidal activity by injection into newly moulted fifth stadium *M. brassicae* larvae. Each 5µl injection contained a total of 50µg of fusion protein (quantified by SDS-PAGE) re-suspended in PBS. Injections of PBS were also carried out as a negative control.

Both recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin injected larvae showed 100% mortality in 48 hours (Figure 4.38). However, no paralysis was observed in insects injected with either fusion protein. A survival level of 95% was observed in the PBS negative control injections (Figure 4.38).

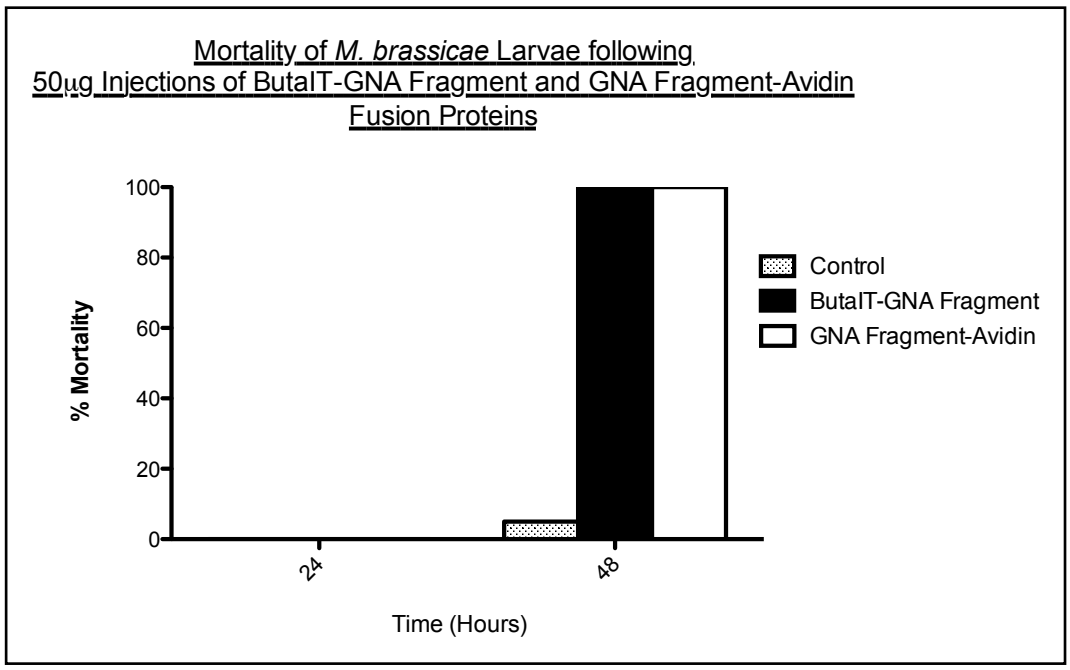

Figure 4.38.

Mortality of *M. brassicae* larvae following injection with 50µg of recombinant ButaIT-GNA Fragment or GNA Fragment-Avidin fusion protein.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

An attempt was made to establish the lowest effective dose (causing mortality) of both fusion proteins. However, 100% mortality was observed in 48 hours at injection doses as low as 0.05µg.

The oral activity of the recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin fusion proteins was also evaluated by droplet feeding to newly moulted fifth stadium *M. brassicae* larvae. Each 5µl droplet contained a total of 50µg of the fusion protein. Droplets of PBS were fed to larvae as a negative control for the bioassay.

Both recombinant ButaIT-GNA Fragment and GNA Fragment-Avidin were non-toxic when delivered orally. Larvae were completely unaffected by the fusion proteins, with 100% survival and no obvious visual or developmental effects being observed. Similar survival was also observed for the PBS negative control.

The presence of insecticidal activity by injection of the fusion proteins at such low doses and the absence of oral activity were unusual. Also, the absence of paralysis upon injection and the activity of recombinant GNA Fragment-Avidin (not including a toxin) were surprising, suggesting that these fusion proteins were acting on the larvae via a different mechanism to ButaIT-Gavidin.

4. *In vivo* Analysis of Recombinant ButaIT-Gavidin

As a result of the paralysis observed in the recombinant ButaIT-Gavidin droplet feeding assay described on page 131, an investigation into one suggestion that ButaIT-Gavidin may be exerting an effect on the mandibula nerve in the head of *M. brassicae* larvae was conducted. A droplet feeding and dissection experiment was carried out, where starved, newly moulted fifth stadium *M. brassicae* larvae were droplet fed 50μg of either recombinant ButaIT-Gavidin or ButaIT-Gavidin 1 Mutation fusion protein to allow for the analysis of any differences. After 10 minutes, five larvae fed the recombinant ButaIT-Gavidin were rapidly dissected during the paralysis to obtain the head proteins. Guts were also extracted. The larvae fed recombinant ButaIT-Gavidin 1 Mutation were dissected similarly three hours later. Droplets of PBS were fed to larvae as a negative control for the experiment.

Western blotting (anti-avidin antibodies) of the extracted proteins from the head capsule and the gut showed that both of the fusion proteins were degraded *in vivo* (Figure 4.39). Only avidin was detected, suggesting that the degradation of the fusion protein occurs in a matter of minutes and that this would allow the toxin to access any nerve endings, including the mandibula nerve, around the mouth-parts.

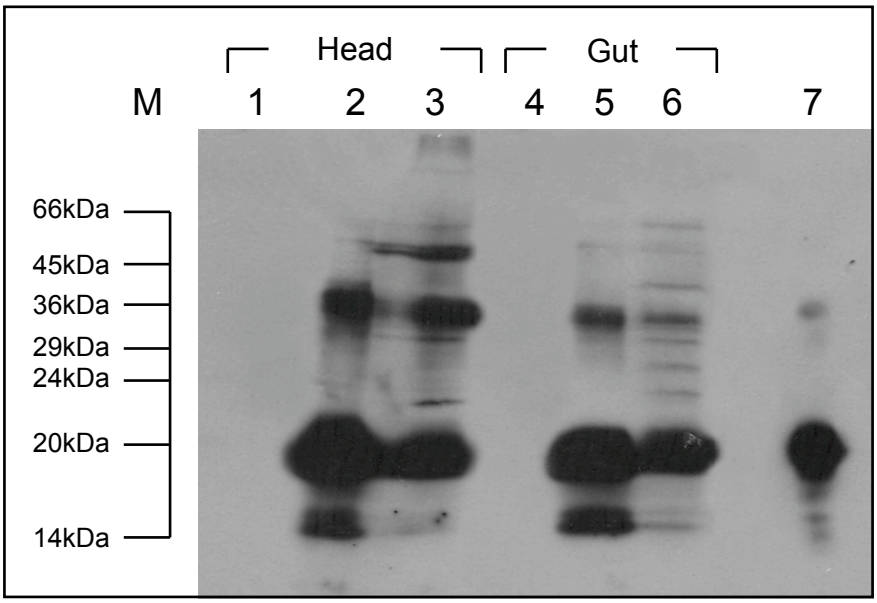

Figure 4.39.

Western analysis of *M. brassicae* larvae head capsules and guts 20 minutes after feeding on a 50µg recombinant ButaIT-Gavidin fusion protein droplet and three hours after feeding on a 50µg recombinant ButaIT-Gavidin 1 Mutation fusion protein droplet.

A one second exposure of the membrane transferred from a 15% SDS-PAGE gel, probed with anti-avidin antibodies (1:10000) is shown. M is SDS 7 molecular weight marker. Lane 1 is head capsules from larvae fed with PBS (control). Lane 2 is head capsules from larvae fed with ButaIT-Gavidin for 20 minutes. Lane 3 is head capsules from larvae fed with ButaIT-Gavidin 1 Mutation for three hours. Lane 4 is guts from larvae fed with PBS (control). Lane 5 is guts from larvae fed with ButaIT-Gavidin for 20 minutes. Lane 6 is guts from larvae fed with ButaIT-Gavidin 1 Mutation for three hours. All of the fusion protein samples show recombinant avidin (20kDa) with some proteolytic degradation (15kDa and 14kDa) and avidin dimerisation (36kDa). (20µl loaded for each sample). Lane 7 is 10ng of recombinant avidin (20kDa).

Following on from this, recombinant ButaIT-Gavidin was fluorescently labeled and a 2µg droplet fed to newly moulted fifth stadium *M. brassicae* larvae on a microscope slide *in situ*, to identify any areas of localised binding within the head capsule. Unfortunately, large amounts of autofluorescence masked any specific binding.

5. Recombinant ButaIT-Gavidin Production Process Analysis

The experiments described above had given no insight into why and how recombinant ButaIT-Gavidin showed high levels of toxicity when injected or delivered orally. The results were both contradictory and confusing. Consequently,

an in depth analysis of the preparation of recombinant ButaIT-Gavidin was carried out to ensure that the insecticidal effects that were observed were not due to contaminants carried over from the production process.

To obtain the correct working concentrations of recombinant ButaIT-Gavidin after lyophilisation, the protein was resuspended in small volumes of PBS solution (50µl-200µl). This would cause problems if the use of such small volumes 'amplified' the concentration of any residual salts that remained after the dialysis step. Imidazole in particular is toxic and is present in the nickel column elution buffer.

To investigate this further, an aliquot of elution buffer equal to the amount contained in a ButaIT-Gavidin column elution was put through a dialysis process identical to that used in preparing recombinant fusion proteins. The elution buffer blank showed some residual salt following lyophilisation, suggesting that the dialysis process was not working as efficiently as first thought. The lyophilised powder obtained from the elution buffer was resuspended in 100µl of PBS in the same way as recombinant ButaIT-Gavidin and 5µl of it was injected into newly moulted fifth stadium *M. brassicae* larvae.

The residual salt showed similar instant paralysis and mortality effects to the recombinant ButaIT-Gavidin fusion protein described on page 130, suggesting that that activity was not entirely due to the fusion protein, but also partly to the residual salt remaining within the protein sample.

To establish the actual activity of the recombinant ButaIT-Gavidin fusion protein without the contaminants, an existing 2mg batch of the fusion protein was desalted using a PD10 desalting column and subsequently 50µg and 100µg was injected into newly moulted fifth stadium *M. brassicae* larvae. Injections of PBS were also carried out as a negative control.

The desalted recombinant ButaIT-Gavidin fusion protein showed no insecticidal activity (Figure 4.40). The injected *M. brassicae* larvae were completely

unaffected by the injected fusion protein, with 100% survival and no obvious visual or developmental effects being observed. Similar survival was observed in the PBS negative control injections (Figure 4.40).

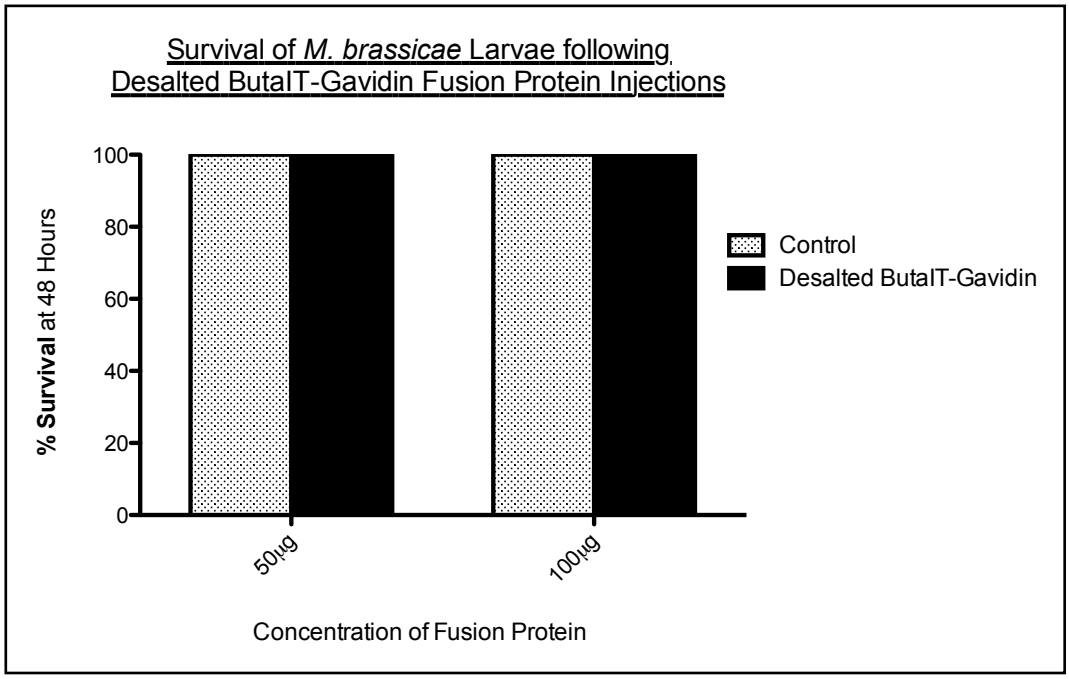

Figure 4.40.
Survival of *M. brassicae* larvae following injection with varying amounts of desalted recombinant ButaIT-Gavidin fusion protein.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

To confirm this result, a further batch of recombinant ButaIT-Gavidin fusion protein was produced by fermentation and purification as described on page 129, but more extensive dialysis was carried out over three days to ensure all of the elution buffer salts were removed. Following lyophilisation and fusion protein content analysis by SDS-PAGE, injections of 50µg and 100µg of the recombinant ButaIT-Gavidin were carried out in newly moulted fifth stadium *M. brassicae* larvae. Injections of PBS were also carried out as a negative control.

As with the desalted recombinant ButaIT-Gavidin, no activity was observed (100% survival in all cases), showing that the recombinant ButaIT-Gavidin fusion protein was not insecticidal and residual salts in the sample caused the results described on pages 130, 131 and 132.

6. Insecticidal Activity of Imidazole

If the nickel column imidazole elution buffer was to be used in further studies, it was important to establish the exact toxicity of imidazole to *M. brassicae* larvae. Newly moulted fifth stadium *M. brassicae* larvae were injected with 5µl of imidazole re-suspended in PBS at concentrations in the range 0.1mM-300mM. Injections of PBS were also carried out as a negative control.

The imidazole had instant insecticidal effects at concentrations above 1mM, with 50% mortality observed at a concentration of 10mM and 100% mortality observed at a concentration of 100mM (Figure 4.41). A survival level of 100% was observed in the PBS negative control injections (Figure 4.41). From the injections, it can be seen that the LD_{50} of imidazole in *M. brassicae* larvae is a concentration of 10mM.

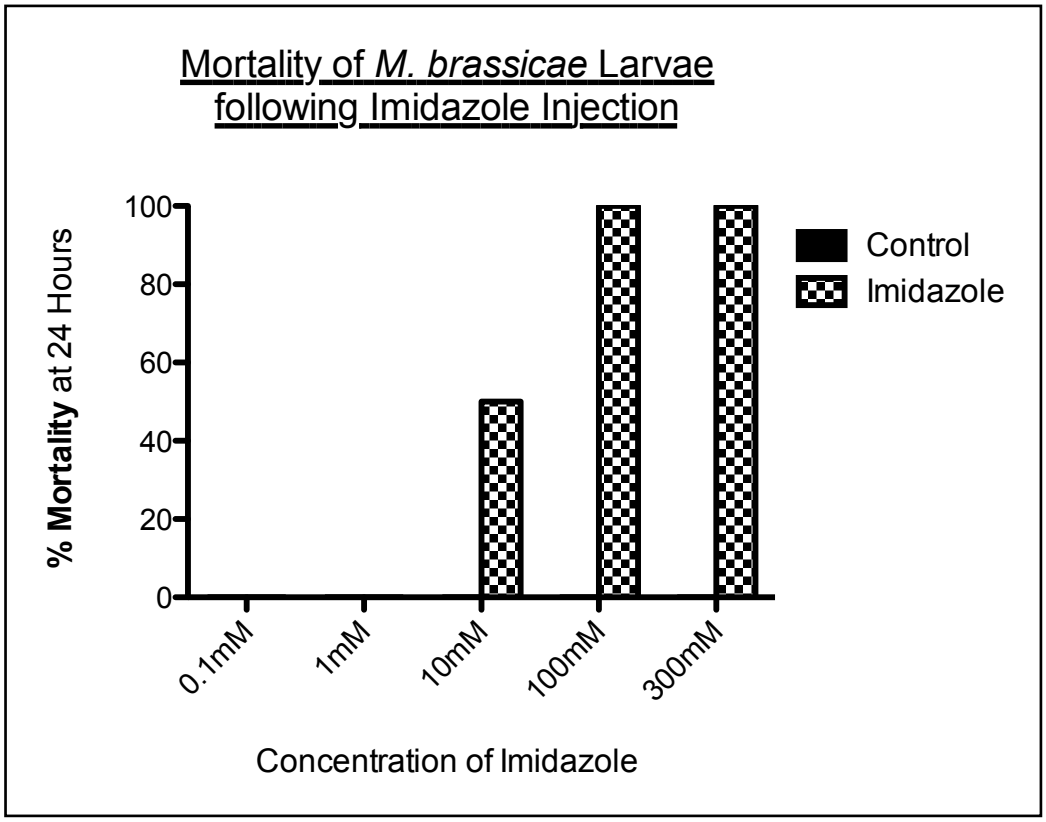

Figure 4.41.

Mortality of *M. brassicae* larvae following injection with varying concentrations of imidazole.

(n = 20 for each concentration - Two separate treatments with 10 individuals).

Discussion

This chapter presents the experiments carried out in an attempt to produce an insecticidal recombinant avidin fusion protein in *Pichia pastoris*.

The three recombinant Avidin-ButaIT fusion proteins (Figures 4.1, 4.2 and 4.3) produced initially are based on existing GNA fusion proteins (Pham-Trung *et al.*, 2006). All three show similar expression levels in SMD1168 *P. pastoris*. Although this is the protease deficient strain of *P. pastoris*, some proteolytic degradation is still observed (Figure 4.6). This is because the yeast is only deficient in the major proteases and it still requires some protease activity to survive. Unlike the GNA fusion proteins, the recombinant Avidin-ButaIT fusion proteins have no insecticidal activity towards *M. brassicae* larvae even when injected at doses up to 100μg (Figure 4.8). Fusing the ButaIT toxin at the N-terminal or the C-terminal of avidin gives no difference in activity. The interaction with immobilised biotin (Figure 4.10) shows that the avidin within the fusion protein is fully functional. It must therefore be the ButaIT toxin that is inactive, most likely due to incorrect folding of the protein during its production and processing. However, the only functional assay for ButaIT toxin is toxicity when injected into lepidopteran larvae as carried out here. Physical studies to establish whether or not the ButaIT toxin has indeed folded correctly would be a valuable indication of functionality, but the lyophilised fusion proteins are not homogeneous enough for spectroscopic analysis. *In vivo* transport analysis following oral delivery (Figure 4.9) also shows that these fusion proteins are degraded within the gut of the insect, but that avidin (slightly degraded) is still transported and detected in the haemolymph. In contrast, intact GNA fusion protein can be detected in the haemolymph of lepidopteran larvae following oral delivery (Fitches *et al.*, 2004; Pham-Trung *et al.*, 2006). Perhaps another contributing factor to the differences in gut degradation and transport between GNA and avidin fusion proteins could also be the actual mechanism of transport It is well documented that GNA binds to mannose residues in the gut and is subsequently translocated to the haemolymph, however, very little is actually known about avidin transport; is it due to the 'leaky' nature of the lepidopteran gut as described in Chapter 3, or does a more sophisticated method like with GNA exist?

The absence of fusion protein activity and degradation within the gut shows that their design must be researched further.

Mamestra brassicae larvae were unaffected by injection of the recombinant IgG Hinge Avidin-ButaIT fusion proteins (Figures 4.11, 4.12 and 4.14), suggesting that the absence of insecticidal activity might not be due to a spatial constraint on the folding of the ButaIT toxin. The IgG Hinge linker region included here has previously been proven to successfully allow more space for fusion protein component folding in avidin fusions produced by Airenne and Kulomaa (1995). However, this one experiment does not rule out the influence of a spatial constraint on the folding of the ButaIT toxin, as it may be the case that the IgG Hinge linker region only works with the *Schistosoma japonicum* glutathione S-transferase fusion protein (Airenne and Kulomaa, 1995). It is possible that the IgG Hinge linker region is too short, too long or even biochemically incompatible to maintain the correct folding of the ButaIT toxin. Degradation analysis (Figure 4.15) shows that the recombinant IgG Hinge Avidin-ButaIT fusion proteins are stable within the haemolymph of the insect. This confirms that the absence of insecticidal activity of the recombinant fusion proteins is not due to degradation of the ButaIT toxin and again supports the fact that the ButaIT toxin component of the fusion protein may still be incorrectly folded. However, this does not rule out the possibility that the recombinant IgG Hinge Avidin-ButaIT fusion proteins are too stable within the haemolymph of the insect, even though similar enzyme restriction sites to those utilised in GNA fusion proteins were used. Of course, avidin could also hinder the target interaction or direct the toxin away from the target completely. This would require further investigation.

The recombinant ωACTXHv1a-Avidin fusion protein (Figure 4.16) produced subsequently does not show insecticidal activity towards *M. brassicae* larvae either (Figure 4.18). This clearly establishes that using an alternative toxin has no effect on the activity of the fusion protein, presumably as this toxin is also incorrectly folded.

Although non-toxic, all six of these fusion proteins express with only slightly lower yields in comparison to the corresponding GNA fusion proteins, where insecticidal activity is observed (Pham-Trung *et al.*, 2006). The most accurate

method of protein quantification in this case is SDS-PAGE (Figure 4.7). Although it is known that different proteins stain differently, other methods such as bicinchoninic acid assays only give total protein quantification. Analysis by SDS-PAGE allows the actual fusion protein content to be quantified. In this investigation, the aim was to produce and test the fusion proteins for insecticidal activity. However, where fusion proteins are eluted as a single peak from a nickel column (Figure 4.4), it is likely that the elution peak contains a mixture of his-tagged fusion proteins due to proteolytic degradation. Quantification by SDS-PAGE would not distinguish between these variants if the proteolysis did not result in a significant change in molecular weight, for example, removal of a single amino acid from the N-terminus.

The attempt to produce a non-toxin avidin fusion protein (Figure 4.19), to establish if the functionality of a protein is retained following fusion to avidin, was also unsuccessful.

The conclusion following these attempts is that the component fused to the avidin is not being processed correctly. Avidin may actually be interfering with the folding. The toxins do however appear to be processed correctly in the GNA based fusion proteins: GNA may even promote the correct folding of the fused components.

Hence, the final attempt at producing an avidin fusion protein incorporated a 17 amino acid fragment of GNA as a linker. This is based on structural analysis of the Avidin-ButaIT fusion protein and the corresponding GNA fusion protein (Figure 4.22). The GNA fragment was included to reverse the charge of the protein between the ButaIT toxin and the avidin to make it similar to that contained within a GNA fusion protein (Figures 4.23 and 4.24).

The recombinant ButaIT-Gavidin fusion protein showed some degradation during production (Figure 4.25), but following injection and droplet feeding in *M. brassicae* larvae (Figures 4.26 and 4.27), it appeared to be active. The paralysis observed after feeding a single droplet looked identical to that which would be associated with a toxin, but the insecticidal activity was ten times greater than that of the GNA fusion proteins on a per amount of protein basis.

Subsequently, the mode of action of the recombinant ButaIT-Gavidin fusion protein was analysed. Protein N-terminal sequencing showed that the cleavage of recombinant ButaIT-Gavidin during production in *P. pastoris* was due to one or more chymotrypsin-like protease (Figure 4.29 and Table 4.2). Mutation of these cleavage sites increased the yield at the expense of the fusion protein activity (Figures 4.30, 4.31, 4.32, 4.33 and 4.34). The evidence at this point indicated that fusion proteins must be cleaved *in vivo* to be active.

The ButaIT-GNA Fragment and GNA Fragment-Avidin fusion protein experiments show that an insecticidal protein can be produced utilising only the 17 amino acid N-terminal fragment of GNA. An explanation for this is that the fragment of GNA used contains the two relatively hydrophobic beta strands in close proximity to the active binding site and may be able to bind to physiologically active receptors in the insect, resulting in mortality. This is surprising and although these fusion proteins are not orally active following feeding of a single droplet, they may be insecticidal during prolonged exposure in artificial diet. Perhaps further investigation is warranted here as the original ButaIT-GNA fusion protein may not require the complete GNA protein sequence to be orally active.

Based on the evidence from the injection and feeding bioassay experiments described earlier, it is difficult to provide a scientific explanation of the activity of the recombinant ButaIT-Gavidin fusion protein. Fluorescence microscopy and western blotting (Figure 4.39) show no convincing evidence as to the mode of action of this fusion protein. No differences in degradation are observed between the recombinant ButaIT-Gavidin and the recombinant ButaIT-Gavidin 1 Mutation (Figure 4.39), although the recombinant ButaIT-Gavidin 1 Mutation is active over 48 hours when compared to recombinant ButaIT-Gavidin without mutation (less than 24 hours) following injection into *M. brassicae* larvae (Figure 4.33).

Process analysis shows that the initial conclusion of the existence of an active fusion protein is a mistake. The observed activity is due to the presence of imidazole that is included in the nickel column elution buffer and has not dialysed out efficiently, even though dialysis was carried out following the manufacturer recommended procedure. Protein elution peaks containing 300mM imidazole elution

buffer were initially diluted 1 in 10 before dialysis in 10 litres of purified water. The water was refreshed twice, at three-hour intervals, with the final change being left overnight (a total of 24 hours dialysis). Calculations show that the concentration of imidazole in the protein sample should be reduced to 30nM in that time, if the dialysis proceeds to completion. When resuspended in 100μl of PBS, the concentration of imidazole in the protein sample would therefore have been 0.3μM. However, the presence of salts in the lyophilised buffer sample alone shows that for the nickel column elution buffer, the dialysis process works less efficiently.

In order to avoid the same problem in future studies, the insecticidal activity of imidazole is presented in Figure 4.41. All dialysis and resuspension following lyophilisation must ensure that the imidazole concentration is at 1mM or below. This would have been the case if the dialysis had worked at maximum efficiency. Moreover, the effects of imidazole on *M. brassicae* larvae appeared to be similar to those of a toxin (paralysis of the hind abdominal and anal prolegs).

In conclusion, none of the attempts to produce an active recombinant avidin fusion protein in *P. pastoris* were successful: avidin seems unsuitable for use as a 'carrier' based on these cases of synthetic fusion protein technology.

Chapter 5

Production of Recombinant Avidin Conjugates

Introduction

Avidin can form stable conjugates via its biotin-binding sites (Chapter 1). Avidin conjugates are not limited to those with biotin itself, but can include a range of compounds to which biotin has been chemically linked. These will bind via the biotin-binding sites on avidin. This is the basis for the biotin-agarose interaction with avidin described in Chapter 3. Binding biotin-linked compounds to avidin is an alternative, practical method of forming fusions because of the very strong interaction between avidin and biotin. The availability of methods for producing biotinylated peptides and proteins allows a range of novel conjugates to be produced. There are no records of insecticidal avidin conjugates in the scientific literature.

Previous research has shown that biotin remains bound to avidin following transfer to the haemolymph of *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae (Hinchliffe, 2007). This study also shows that avidin behaves differently in aphids, it being held in the gut instead of being transported to the haemolymph (Chapter 3). This information should allow specific toxins to be chosen for the different insect species.

The experiments in this chapter describe attempts to produce an insecticidal avidin conjugate as an alternative to 'conventional' fusion proteins (Chapter 4). Whilst the synthetic fusion proteins contain components linked by covalent peptide bonds in a single polypeptide chain, the avidin conjugates will contain components linked by the non-covalent avidin-biotin binding interaction. Beginning with aphids, where avidin is held in the gut and not transported, a conjugate with the leucomyosuppressin (LMS) neuropeptide from *Leucophaea maderae* (cockroach) (Orthoptera: Blaberidae, Fabricius 1781) will be produced. Specifically, LMS inhibits muscular gut contractions, making it an ideal initial candidate as aphids rely heavily on gut motility for digestion and therefore survival. Peptide toxins such as

ButaIT from *Mesobuthus tamulus* (Indian red scorpion) (Scorpiones: Buthidae, Fabricius 1798) utilised in Chapter 4 would not be suitable for use on aphids as the toxin target lies on the haemolymph side of the gut, where it is established avidin does not reach (Chapter 3). Finally, a conjugate with the allatostatin neuropeptide from *Manduca sexta* (tobacco hornworm) (Lepidoptera: Sphingidae, Linnaeus 1763) will be produced and analysed for activity on lepidopteran larvae (*M. brassicae*).

Results

Conjugation to Avidin Causes a Peptide to be Retained in the Hemipteran Gut

To determine whether a molecule conjugated to avidin was retained in the aphid gut just as avidin alone is (Chapter 3), fluorescently labelled biotin (fluorescein-biotin) and a fluorescently labelled, biotinylated peptide (biotin-Cys[AlexaFluor488]-Leucomyosuppressin) were conjugated to avidin (Figure 5.1).

The *Acyrthosiphon pisum* (pea aphid) (Hemiptera: Aphididae, Harris 1776) genome contains a predicted gene encoding a precursor to leucomyosuppressin (LMS) (LOC100163798), which is translated into a 1077 nucleotide mRNA. The predicted hormone protein sequence from this gene is QDLDHVFLRF-amide, where the C-terminal amidation is inferred from the presence of a C-terminal -GRRRR sequence. The N-terminal of the hormone is predicted by a dibasic residue cleavage sequence, -KR-. This protein sequence was not available when the experiment was designed, and a peptide sequence similar to the LMS peptide isolated from *Leucophaea maderae* (cockroach) (Orthoptera: Blaberidae, Fabricius 1781) (Chapter 1), with the exception that the N-terminal glutamic acid residue was replaced with glutamine (substitution E1Q), was used. This differs from the *A. pisum* LMS sequence only by a single amino acid substitution near the N-terminus (V3L), which is unlikely to have a major effect on biological activity in aphids, since it is the C-terminal regions of FLRFamide peptides in insects that are known to be critical for activity. The peptide was synthesised with an extra N-terminal cysteine residue, and labelled on the cysteine side chain with a fluorophore (AlexaFluor488) (product: Cys[AlexaFluor488]-QDVDHVFLRF-amide). A biotin residue was also added to the N-terminus of the peptide to allow affinity conjugation to avidin. The resulting peptide (biotin-Cys[AlexaFluor488]-QDVDHVFLRF-amide) and fluorescein-biotin were conjugated to avidin by incubation in molar excess (2:1), followed by dialysis to remove the unbound biotin or peptide.

The labelled conjugates and controls (unconjugated fluorescein-biotin and biotin-Cys[AlexaFluor488]-LMS) were included in separate treatments of artificial diet at a concentration of 0.1mg/ml (100ppm, 100μM) and fed to *A. pisum* for 48 hours. Half of the aphids then received a chase of diet containing no added

compounds for 24 hours. Propidium iodide was also included in the artificial diet at a concentration of 0.1mg/ml to fluorescently stain DNA, which is present at the highest concentration within the gut cells, thereby outlining the gut of the aphids. Following the feeding treatments, whole aphids were viewed on a fluorescence microscope and images were captured in Openlab (see Chapter 2) (Figure 5.2).

As expected, all of the labelled compounds and conjugates were detected throughout the gut when fed, but their retention after the chase period differed according to the compound. Identical to the results obtained for feeding fluorescein isothiocyanate (FITC) label alone, fluorescein-biotin was not retained in the aphid and disappeared during the chase period. On the other hand, the avidin : fluorescein-biotin conjugate was retained in the aphid foregut region. Unconjugated biotin-Cys[AlexaFluor488]-LMS peptide was removed from the aphid during the chase period, but the avidin : biotin-Cys[AlexaFluor488]-LMS conjugate was retained in the aphid foregut region after the chase (Figure 5.2). These results show that conjugating a peptide to avidin will cause it to be retained in the aphid, as a result of the avidin binding to the stomach region of the gut.

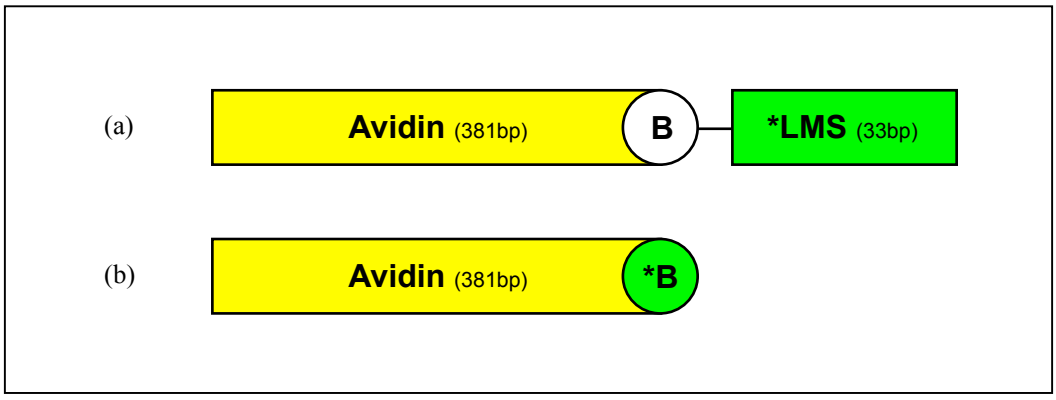

Figure 5.1.

Schematic representation of the fluorescent avidin conjugates.
(a) Avidin : biotin-Cys[AlexaFluor488]-LMS. (b) Avidin : fluorescein-biotin.
Diagram not to scale.

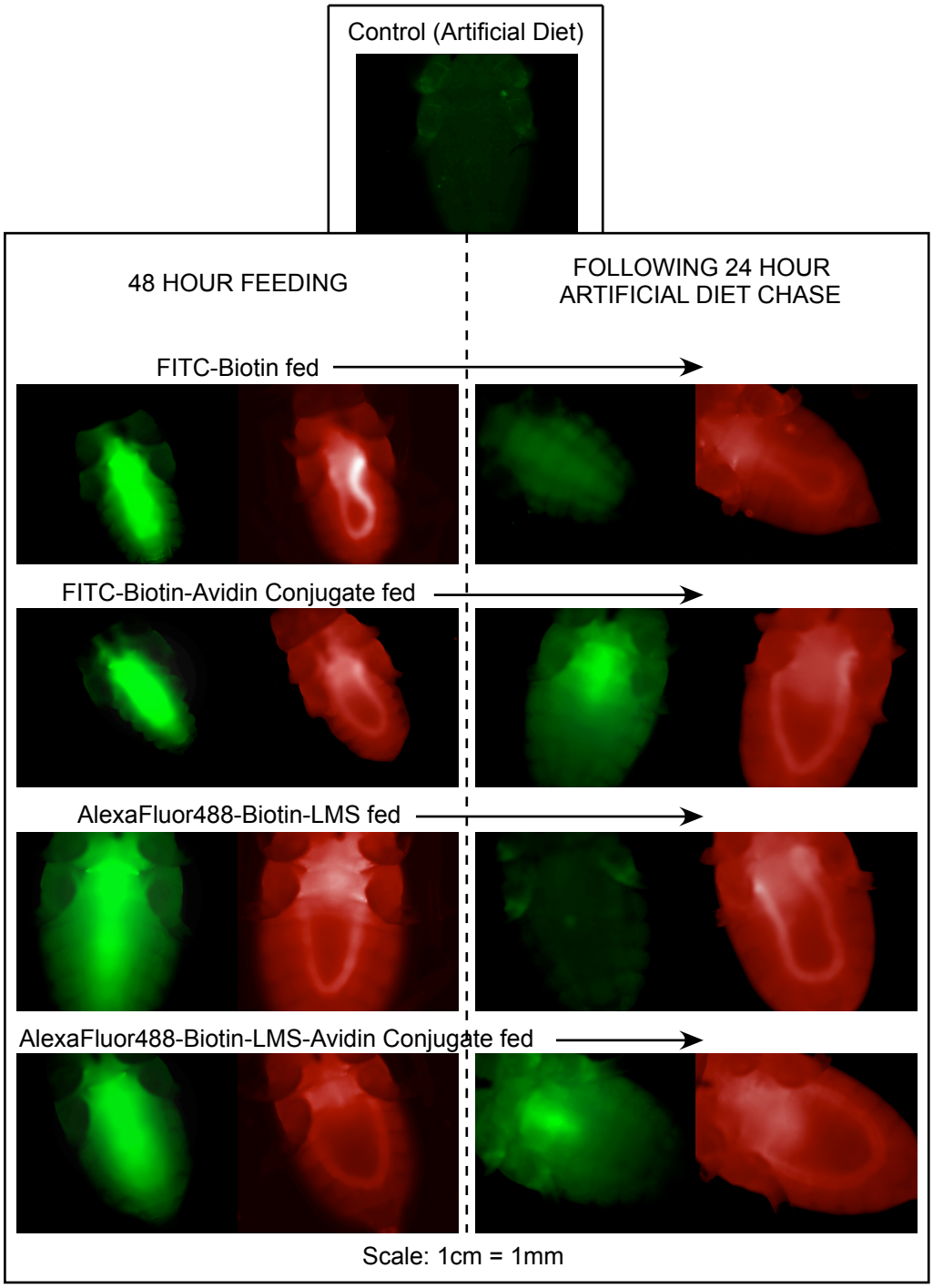

Figure 5.2.

Fluorescence micrographs of *A. pisum* (pea aphid) after feeding on 0.1mg/ml fluorescein-biotin, avidin : fluorescein-biotin, biotin-Cys[AlexaFluor488]-LMS or avidin : biotin-Cys[AlexaFluor488]-LMS.

(Left hand side) After feeding for 48 hours. (Right hand side) After chasing fluorescent label with control diet for 24 hours. Green channel images show fluorescence from labelled compounds. Red channel images show fluorescence from propidium iodide used as a general stain.

Preparation and Insecticidal Activity of an Avidin : biotin-LMS Conjugate in Hemiptera

If avidin binding can be used to 'anchor' conjugated molecules at the gut surface in the aphid stomach, the resulting high local concentrations of a potential toxin could increase its insecticidal activity. This hypothesis was tested by preparing a conjugate between avidin and N-terminally biotinylated LMS (avidin : biotin-LMS, with the LMS being the QDVDHVFLRF-amide identical to that described on page 161) (Figure 5.3).

Avidin is insecticidal to *A. pisum* at concentrations greater than 0.25mg/ml (250ppm, 3.8μM) (Chapter 3). A sub-lethal concentration of 0.1mg/ml avidin (100ppm, 1.6μM) in artificial diet was therefore selected, at which more than 60% of pea aphids survived from neonate to maturity (10 days) compared to control survival of 90%-100%. Biotin-LMS was non-toxic to *A. pisum* at concentrations up to 0.25mg/ml (250ppm; highest tested), with no significant differences to controls. The avidin : biotin-LMS conjugate was prepared by incubation of avidin with a molar excess (2:1) of biotin-LMS, followed by removal of the excess peptide by dialysis (Figure 5.4). To compare their insecticidal effects, avidin, biotin-LMS, and avidin : biotin-LMS conjugate were fed to *A. pisum* at the same concentration in artificial diet (0.1mg/ml, 100ppm) and the effects on survival were recorded. Survival curves are presented in Figure 5.5.

Over the 12-day bioassay, survival of the control and LMS fed aphids was ≥90%. Survival of the avidin-fed aphids declined to 70% by day 12 and they showed some growth retardation as expected. In contrast, aphids fed the avidin : biotin-LMS conjugate showed a continuous decline in survival throughout the bioassay, with survival down to 25% by day 12 (Figure 5.5). Comparison of the survival curves shows that survival for the aphids fed diet containing avidin : biotin-LMS conjugate was statistically different from the survival of the aphids fed with any other treatment (Mantel-Cox log-rank, $P<0.001$). The other survival curves did not differ significantly from each other, with the exception of the aphids fed avidin, which differed marginally from the control (Mantel-Cox log-rank, $P=0.047$) (Figure 5.5). The data shows that the avidin : biotin-LMS conjugate displays insecticidal activity

towards aphids that is not shown by either of the constituents of the conjugate on their own.

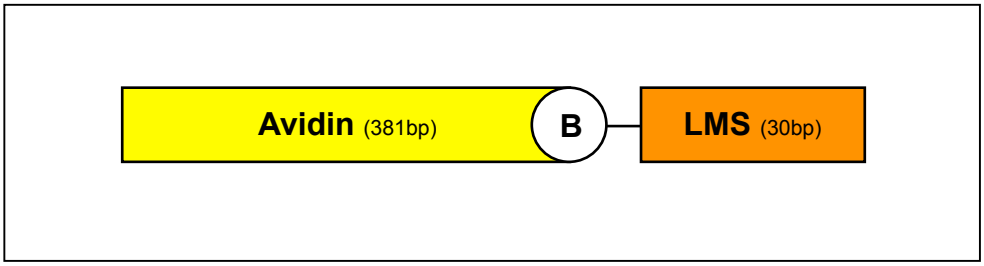

Figure 5.3.

Schematic representation of the avidin : biotin-LMS conjugate.
Diagram not to scale.

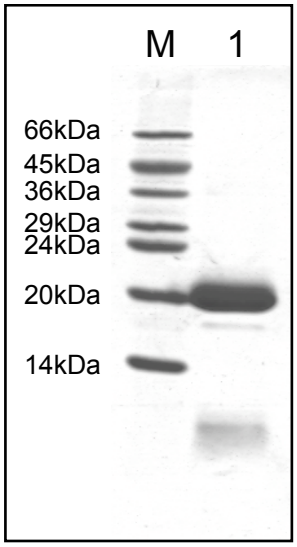

Figure 5.4.

17.5% SDS-PAGE analysis of avidin : biotin-LMS conjugate.
M is SDS 7 molecular weight marker. Lane 1 is 10µl of avidin : biotin-LMS conjugate. The conjugate dissociates upon boiling, showing both the avidin (20kDa) and LMS (1.3kDa) components.

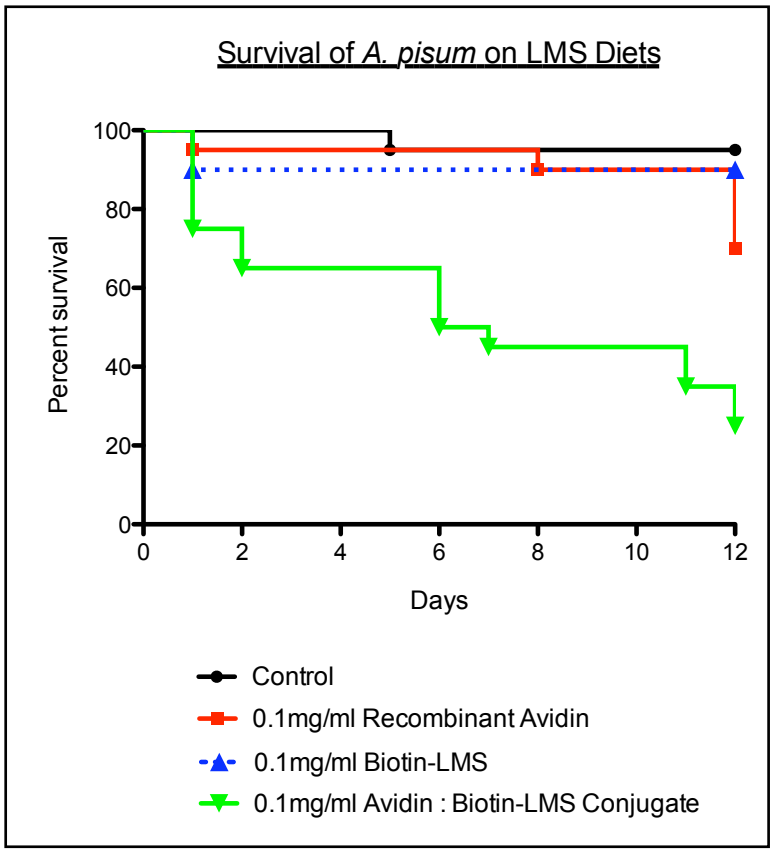

Figure 5.5.

Survival of *A. pisum* fed on artificial diet containing 0.1mg/ml recombinant avidin, biotin-LMS or avidin : biotin-LMS conjugate.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

Preparation, Insecticidal Activity and Transport of an Avidin : biotin-allatostatin Conjugate in Lepidoptera

As an avidin-biotin conjugate is transported to the haemolymph in lepidoptera (Hinchliffe, 2007), the hypothesis that a biotinylated peptide could be conjugated to avidin and similarly transported, possibly to show insecticidal activity, was tested. A conjugate between avidin and N-terminally biotinylated allatostatin (avidin : biotin-allatostatin) was used as the subject for assays (Figure 5.6).

Allatostatin, similar to that isolated from *Manduca sexta* (tobacco hornworm) (Lepidoptera: Sphingidae, Linnaeus 1763) (Chapter 1), was synthesised with a biotin residue added to the N-terminus of the peptide to allow affinity conjugation to

avidin. The resulting peptide (biotin-QVRFRQCYFNPISCF) was conjugated to avidin by incubation in molar excess (2:1), followed by dialysis to remove the unbound peptide (Figure 5.7).

Initially, to check the activity of the biotin-allatostatin and avidin : biotin-allatostatin conjugate, 5µl injections of amounts between 1µg and 10µg were carried out in newly moulted fifth stadium *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae. These amounts were based on the injections carried out by Audsley *et al.* (2001) in *Lacanobia oleracea* (tomato moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae. As biotin-allatostatin was resuspended in 10% (v/v) Dimethyl suphoxide (DMSO), injections of 5µl of 10% DMSO were carried out as a negative control.

At the 10µg dose, both the biotin-allatostatin and avidin : biotin-allatostatin conjugate gave 100% mortality in 48 hours, whereas the DMSO negative control larvae showed 100% survival (Mantel-Cox log-rank statistical analysis, $P<0.0001$) (Figure 5.8). At the 5µg dose, the biotin-allatostatin gave 80% mortality and the avidin : biotin-allatostatin conjugate gave 50% mortality in 48 hours, both of which are statistically significant (Mantel-Cox log-rank statistical analysis, compared to control, $P=0.0006$ and $P=0.0162$ respectively). At the 1µg dose, 100% survival was observed for both samples (Figure 5.8). These results suggest that both the biotin-allatostatin and avidin : biotin-allatostatin conjugate are active.

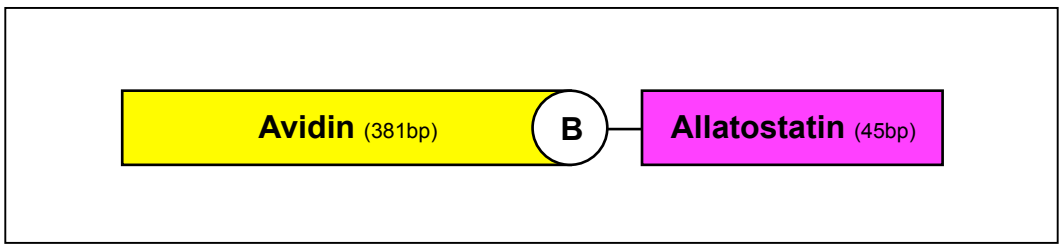

Figure 5.6.

Schematic representation of the avidin : biotin-allatostatin conjugate.
Diagram not to scale.

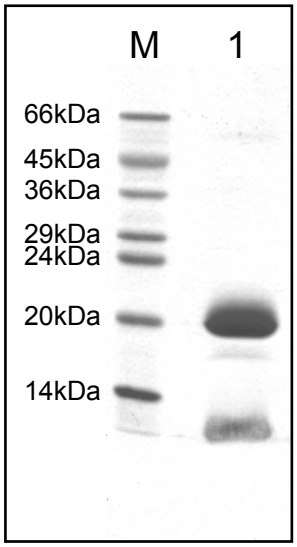

Figure 5.7.

17.5% SDS-PAGE analysis of avidin : biotin-allatostatin conjugate.

M is SDS 7 molecular weight marker. Lane 1 is 10µl of avidin : biotin-allatostatin conjugate. The conjugate dissociates upon boiling, showing both the avidin (20kDa) and allatostatin (2kDa) components.

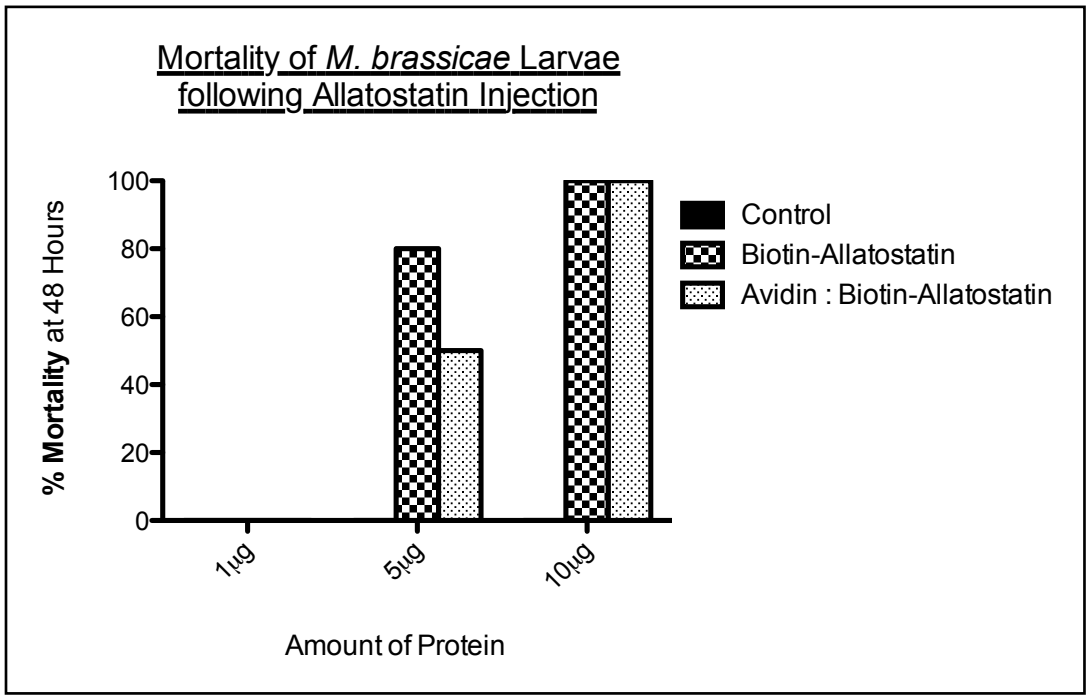

Figure 5.8.

Mortality of *M. brassicae* larvae following injection with varying amounts of biotin-allatostatin and avidin : biotin-allatostatin conjugate.

(n = 20 for each treatment - Two separate treatments with 10 individuals).

The next step was to look for insecticidal activity when the avidin : biotin-allatostatin conjugate was fed to *M. brassicae* larvae. The avidin : biotin-allatostatin conjugate was fed at a dose of 1000ppm (5mg conjugate in 5g artificial diet), an amount of allatostatin well in excess of that fed by Matthews *et al.* (2008). However, the insecticidal activity of avidin alone towards *M. brassicae* larvae when included at 1000ppm in artificial diet (5mg in 5g diet) had previously been established (Hinchliffe, 2007). This insecticidal activity of avidin needed to be taken into account when determining the insecticidal effect of the conjugate containing avidin. Thus, a separate treatment of recombinant avidin (1000ppm, 5mg in 5g artificial diet) was fed to neonate *M. brassicae* larvae alongside avidin : biotin-allatostatin conjugate (1000ppm, 5mg in 5g artificial diet). Any effects deviating from those of avidin would be due to the allatostatin component of the conjugate. A biotin-allatostatin (1000ppm, 5mg in 5g artificial diet) and a diet-only treatment were also included as negative controls. Survival was recorded over 20 days for all treatments.

The avidin : biotin-allatostatin conjugate had no significant effects on the survival of the *M. brassicae* larvae as only 5% mortality was observed (Figure 5.9). The recombinant avidin treatment showed 100% mortality spread over the 20-day bioassay. The diet-only control and biotin-allatostatin showed 100% survival. This suggests that the avidin : biotin-allatostatin conjugate is not insecticidal.

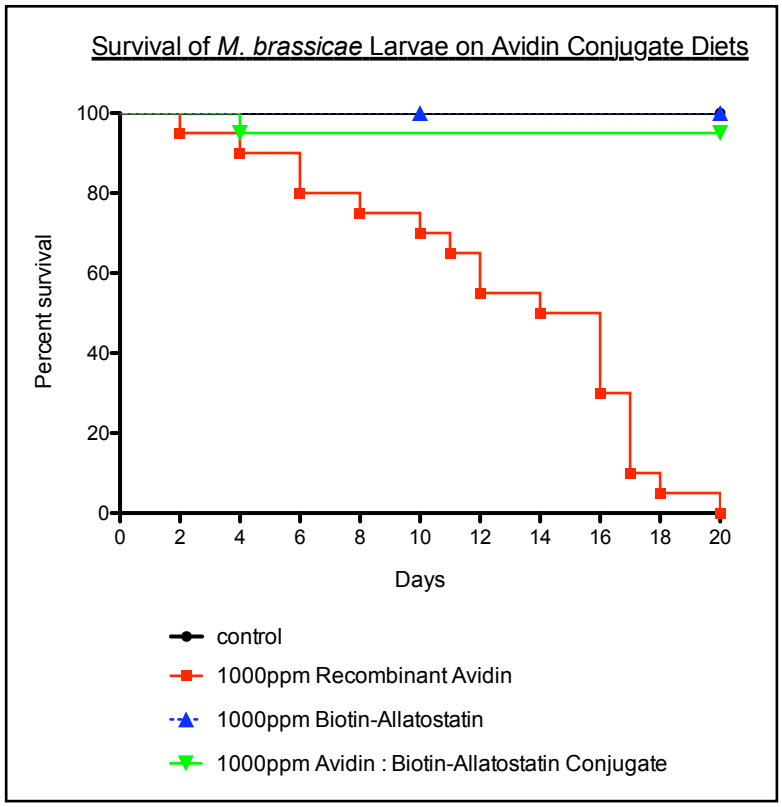

Figure 5.9.

Survival of *M. brassicae* larvae fed on artificial diet containing 1000ppm recombinant avidin, biotin-allatostatin or avidin : biotin-allatostatin conjugate.
(n = 20 for each treatment - Two separate treatments with 10 individuals).

To investigate the *in vivo* transport of the avidin : biotin-allatostatin conjugate, *M. brassicae* larvae from the above experiment were allowed to continue feeding on 1000ppm of avidin : biotin-allatostatin conjugate until they reached fifth stadium. At this point, haemolymph was extracted from three larvae, pooled together and analysed by western blotting (anti-allatostatin and anti-avidin antibodies). Haemolymph from larvae fed with artificial diet only was included as a control (Figure 5.10).

The results show the presence of two bands in the avidin : biotin-allatostatin conjugate positive control (20kDa and 2kDa), indicating that the conjugate had not dissociated completely into avidin and biotin-allatostatin upon boiling in preparation for the blot (Figure 5.10(a)). Hence, it can be seen that there is an absence of avidin :

biotin-allatostatin conjugate within the haemolymph of the *M. brassicae* larvae. There is also an absence of any free biotin-allatostatin within the haemolymph of the larvae (Figure 5.10(a)), suggesting that the conjugate is degraded before it reaches the haemolymph. However, the remaining avidin (19kDa) was transported and readily detected in the haemolymph (Figure 5.10(b)).

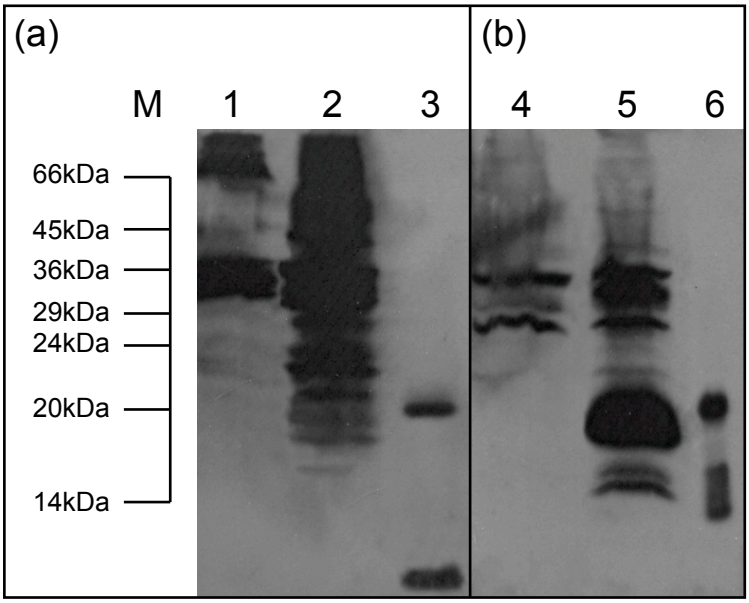

Figure 5.10.

Western analysis of *M. brassicae* extracted haemolymph after feeding on 1000ppm avidin : biotin-allatostatin conjugate.

One second exposures of the membrane transferred from a 15% SDS-PAGE gel. M is SDS7 molecular weight marker. (a) Anti-allatostatin antibody (1:5000). Lane 1 is haemolymph from larvae fed with artificial diet (control). Lane 2 is haemolymph from larvae fed with avidin : biotin-allatostatin conjugate. (25µl loaded for each sample). Lane 3 is 3µg of avidin : biotin-allatostatin conjugate, showing the conjugate (20kDa) and unconjugated biotin-allatostatin (2kDa). The presence of two bands indicates the conjugate has not completely dissociated into avidin and biotin-allatostatin upon boiling in preparation for the blot. (b) Anti-avidin antibody (1:10000). Lane 4 is haemolymph from larvae fed with artificial diet (control). Lane 5 is haemolymph from larvae fed with avidin : biotin-allatostatin conjugate, showing the presence of avidin (19kDa). (25µl loaded for each sample). Lane 6 is 10ng of avidin : biotin-allatostatin conjugate (20kDa).

Discussion

This chapter presents the experiments carried out with the aim of producing an insecticidal avidin conjugate.

It clearly demonstrates that conjugation of a peptide to avidin causes the resultant conjugate to be retained within the aphid gut (Figure 5.2).

The toxicity of the avidin : biotin-LMS conjugate when fed to *A. pisum* observed in the experiments described in this chapter is comparable to that observed in previous experiments reported by Wiles (2007). In these earlier experiments, the retention of avidin in the aphid stomach region after feeding was not demonstrated and therefore the basis for the toxicity of the conjugate was not established.

The results presented here (Figures 5.5 and 5.2) also establish that the toxicity of the avidin : biotin-LMS conjugate to *A. pisum* is due to the retention of the peptide in the stomach, as a result of conjugation to avidin. Proteolytic activity in the pea aphid gut is low (Hinchliffe *et al.*, 2010) and although protection of the peptide from proteolysis cannot be ruled out, the role of the avidin is more likely to be that of anchoring a high concentration of peptide at the gut surface. The absence of biotin-LMS binding in the gut of the aphid dismisses the suggestion in Chapter 3 that binding of avidin in hemiptera is mediated through biotin.

In contrast to the results observed in *A. pisum*, the avidin : biotin-allatostatin conjugate is not orally insecticidal to *M. brassicae* larvae (Figure 5.9). One point to be noted from the feeding experiment is that the insecticidal effects of avidin are also abolished upon conjugation with biotin-allatostatin. The biotin-binding sites are saturated by the conjugated biotin-allatostatin, hence no dietary biotin can be sequestered. This evidence also supports the toxicity of the avidin : biotin-LMS conjugate in *A. pisum* is due to the conjugated biotin-LMS and not as a result of any toxicity from the avidin.

Further analysis shows there is an absence of any avidin : biotin-allatostatin conjugate or free biotin-allatostatin within the haemolymph of the larvae (Figure

5.10). The presence of avidin within the haemolymph shows that the allatostatin is cleaved from the conjugate, most likely by enzymes in the gut prior to transport of the avidin. The cleavage site is most likely to be between the biotin molecule and the allatostatin, because as previously shown, biotin remains bound to avidin upon transfer to the haemolymph (Hinchliffe, 2007). The absence of any allatostatin due to cleavage of the conjugate would explain the absence of any insecticidal effects. Lepidopteran larvae show high levels of gut proteolysis and the degradation of allatostatin in the gut has been documented previously (Audsley *et al.*, 2002). The allatostatin used for these experiments similarly contains arginine residues that may be susceptible to trypsin-like serine protease cleavage in the gut of *M. brassicae* larvae. The presence of some immunoreactive, higher molecular weight bands (Figure 5.10) shows that some molecular association may have occurred, which could have affected the conjugate activity. Although this is unlikely to be the cause of the absence of insecticidal activity, it would require further investigation.

In conclusion, the use of avidin for insecticidal conjugates requires further investigation using different biotinylated toxins. However, unless a biotinylated toxin that is resistant to proteolysis can be produced, the results here indicate that the avidin conjugate method may only be suitable for use on insect pests that have lower levels of gut proteolysis, such as hemiptera.

Chapter 6

General Discussion

Herbivorous insect pests are a major constraint on agricultural production. It is estimated that 30-40% of the world's crop produce is destroyed by agricultural pests and associated diseases. In the UK, over 25 million pounds is spent on insecticides, but the withdrawal of many broad-spectrum pesticides and insecticide resistance has prompted the research into more sustainable, protein-based alternatives.

Many insecticidal proteins exist, including those discussed in detail in Chapter 1, but very few protein-based biopesticides have been employed successfully in crop protection. The aim of this project was to investigate the use of recombinant avidin for the protection of crops against insect pests. Recombinant avidin produced in *Pichia pastoris* has now been characterised (Chapter 3, Hinchliffe *et al.*, 2010) and has been shown to have a similar tetrameric structure to egg white avidin. Its functional properties are also similar; the difference in glycosylation that results from expression in the yeast (which can only carry out core N-glycosylation and the addition of mannose residues to carbohydrate side chains on proteins) does not affect the biotin-binding activity of recombinant avidin. Further exploration of this expression system for the production of modified versions of avidin, as suggested by Zocchi *et al.* (2003), would seem justified, as the yields obtained make the production method economically viable for high-value products, if not for a potential biopesticide.

Recombinant avidin feeding bioassays with hemiptera in this study are the first reported and extend the range of insects against which avidin has insecticidal effects. As with the other insects, the cause of the insecticidal activity in *Acyrthosiphon pisum* (pea aphid) (Hemiptera: Aphididae, Harris 1776) is shown to be attributable to sequestration of dietary biotin by the recombinant avidin. However, the retarded growth observed in *Sitobion avenae* (cereal aphid) (Hemiptera: Aphididae, Fabricius 1775) highlights the differences in susceptibility to avidin

between different insect species. Although completely immune insects are rare, the susceptibility towards the biotin-sequestration effects of avidin is largely dependent on the levels of maternally-derived biotin (Markwick *et al.*, 2001; Christeller *et al.*, 2010). This effect could play a role in the differential susceptibility of these two aphid species, since both are parthenogenetic, and the young could readily accumulate biotin from the mother prior to 'birth'; the hypothesis would then be that *S. avenae* embryos accumulate more biotin from the parent aphid than *A. pisum*. An alternative hypothesis, based on the ability of the aphid to 'scavenge' biotin from ingested recombinant avidin, has been advanced earlier (Chapter 3, Hinchliffe *et al.*, 2010). This differential sensitivity has relevance to the possible use of recombinant avidin as a biopesticide, since adaptation of the insect to gain resistance to the pesticide would need to be considered. Like the use of other pesticides, unless the usage of avidin is carefully managed, it is likely that insects could increase the amount of maternally-derived biotin to overcome its insecticidal effects.

A primary finding of the bioassays was that unlike *Mamestra brassicae* (cabbage moth) (Lepidoptera: Noctuidae, Linnaeus 1758) larvae, recombinant avidin is not transported to the haemolymph of *A. pisum* and *S. avenae*, highlighting possible differences in protein transport from the gut to the haemolymph between hemiptera and lepidoptera (Chapter 3, Hinchliffe *et al.*, 2010). Although attempts were made in this study to begin to explain the differences between the two insect orders, they remain largely unknown. It may be possible however, that recombinant avidin transports to the haemolymph of *M. brassicae* due to the 'leaky' nature of the lepidopteran gut. This has been observed with other large proteins (Jeffers and Roe, 2008). In this respect, transport of avidin differs from transport of GNA, which involves the carbohydrate binding activity of the lectin for interaction with the gut surface (Powell *et al.*, 1998). The failure of avidin to transport to the haemolymph of hemiptera, in contrast to GNA, may result from the lack of binding to suitable receptors at the gut surface, although binding to the stomach does occur, as shown by the chase-feed experiment with FITC-labelled recombinant avidin described in Chapter 3. Beyond the time constraints of this project, further investigation of recombinant avidin transport in other insect orders may highlight trends in the differences and help to explain exactly how avidin transport works in nature. There is also currently renewed interest in elucidating the exact avidin binding and

transportation mechanism. Evidence from past experiments shows that avidin associates with condensed chromatin, mast cell granules and heparin (Heggeness, 1977; Tharp *et al.*, 1985; Kett *et al.*, 2003). Just recently, avidin has been shown to bind to adenosine (Bing *et al.*, 2012). These new developments may also help to explain the results obtained in this study. Perhaps an addition may be to develop a yeast two-hybrid system.

The use of avidin in synthetic fusion protein technology proved unsuccessful (Chapter 4). Evidence suggests that the fused peptide component folded incorrectly in *Pichia pastoris* when attached covalently to avidin. Similar results were also observed with garlic lectin (*Allium sativum* agglutinin; ASA) (Fitches *et al.*, 2008). Consistent with this idea is the observation that toxin sequences themselves fold poorly in *P. pastoris*, since their insecticidal activity is much lower than literature values for toxins purified from natural sources (data not presented). Avidin may further inhibit the folding process, resulting in products that have no insecticidal activity at all. Cereghino and Cregg (2000) noted that when using *P. pastoris* as an expression host, the resultant glycosylation can interfere with protein folding, especially any attached proteins. This study shows that recombinant avidin expressed in *P. pastoris* is glycosylated. Therefore, perhaps a further investigation would involve attempting to mutate and replace the asparagine residue glycosylation site (amino acid number 25 including the N-terminal extension, Appendix 1) and see if avidin remains functional. If it retains activity, it should be tested again as part of a fusion protein. It may also be useful to look at glycosylation of the toxins utilised here. Extra glycosylation by the *P. pastoris* expression host may also affect their folding, not just avidin affecting them. Incorrect folding of the fused peptide was still apparent with an IgG hinge linker region incorporated to increase the spatial distribution between the peptide and avidin. Other claims of insecticidal avidin fusion proteins (Airenne *et al.*, 1999) are limited and show very little evidence of activity or of further processing after their production. Unlike snowdrop lectin (*Galanthus nivalis* agglutinin; GNA), avidin may not be suitable for use as a 'carrier' in synthetic fusion protein technology. A further line of investigation however, before ruling out its usage in the technology, would be to increase the size of the linker, perhaps making it greater than 50 amino acids in length, and study whether or not this produces an active protein. Had time have not been limiting, a more

thorough investigation of the ButaIT-GNA Fragment and GNA Fragment-Avidin fusion proteins would be necessary, as more experimental data is required before any firm conclusions around the use of the 17 amino acid fragment instead of the complete GNA, can be drawn. There is also an important point to note; the GNA Fragment-Avidin fusion protein showed activity by injection, which implies the 51bp GNA Fragment folds correctly (51bp is around half the size of the ButaIT toxin). This being the case, if a toxin that was roughly 50bp in size was utilised in a fusion with avidin and expressed in *P. pastoris*, it should follow that this would also be active. This would require further investigation and although it is not an answer as to why the larger toxin fusion proteins are inactive, it would show if any toxin can be used with avidin at all. The mutational studies (ButaIT-Gavidin 1 Mutation and ButaIT-Gavidin 3 Mutations) in this case gave very little information on the mechanism of fusion proteins as a whole due to the imidazole mistake. However, it may be a useful investigation to try similar mutations with ButaIT-GNA or another GNA fusion protein to determine if chymotrypsin cleavage is required for fusion protein activity in insects. The published literature describes the current use of avidin fusion proteins in medical applications. The majority of these are fusion proteins with antibodies and use different expression hosts (Lesch *et al.*, 2009; Lesch *et al.*, 2010; Suzuki *et al.*, 2010). This shows that active fusion proteins with avidin can be produced, but alternative expression hosts to the *P. pastoris* used in synthetic fusion protein technology may be required. This would require further investigation.

On the other hand, an insecticidal recombinant avidin conjugate was successfully produced (Chapter 5). However, this study found that conjugates of this type may only be useful as an oral insecticide in insects with low levels of gut proteolysis. With more time, further investigation with different insect species would be required to confirm this. Certainly, a further line of enquiry would be to try a conjugate with a different allatostatin without the arginine residue where proteolysis is known to occur before any final conclusions can be made. The option of further biotinylated toxins also exists, but these need to be chosen based on how avidin transports inside the target insect. The scientific literature again describes examples of avidin conjugates used within medical applications (Xia *et al.*, 2009; Zhou *et al.*, 2011), but none show examples of insecticidal conjugates. There will however, be limitations with an insecticidal avidin conjugation method, such as practical

production on a large scale. More cost-effective, alternative methods of peptide biotinylation may be required.

In conclusion, avidin is not currently used for the control of insect pests although the results obtained here, alongside those others reported, show that with management it is clearly an alternative for use alone as a biopesticide. The use of avidin in *P. pastoris* synthetic fusion protein technology was unsuccessful, but this does not rule out its use with other expression hosts. The avidin conjugate method described in this study also shows promising results and potential for insect pest control. Perhaps the key to using avidin as a 'carrier' in avidin fusion proteins and conjugates at all is its actual mechanism of transport in insects compared to GNA.

Finally, the recent literature shows that the avidin-biotin system is still under research and development, with several modified types of avidin now available, including chimeric avidin, Tamavidin, NeutrAvidin and CaptAvidin (Morag *et al.*, 1996; Takakura *et al.*, 2010; Riihimaki *et al.*, 2011). These new, modified varieties of avidin may be useful in future attempts to construct insecticidal fusion proteins and conjugates.

Chapter 7

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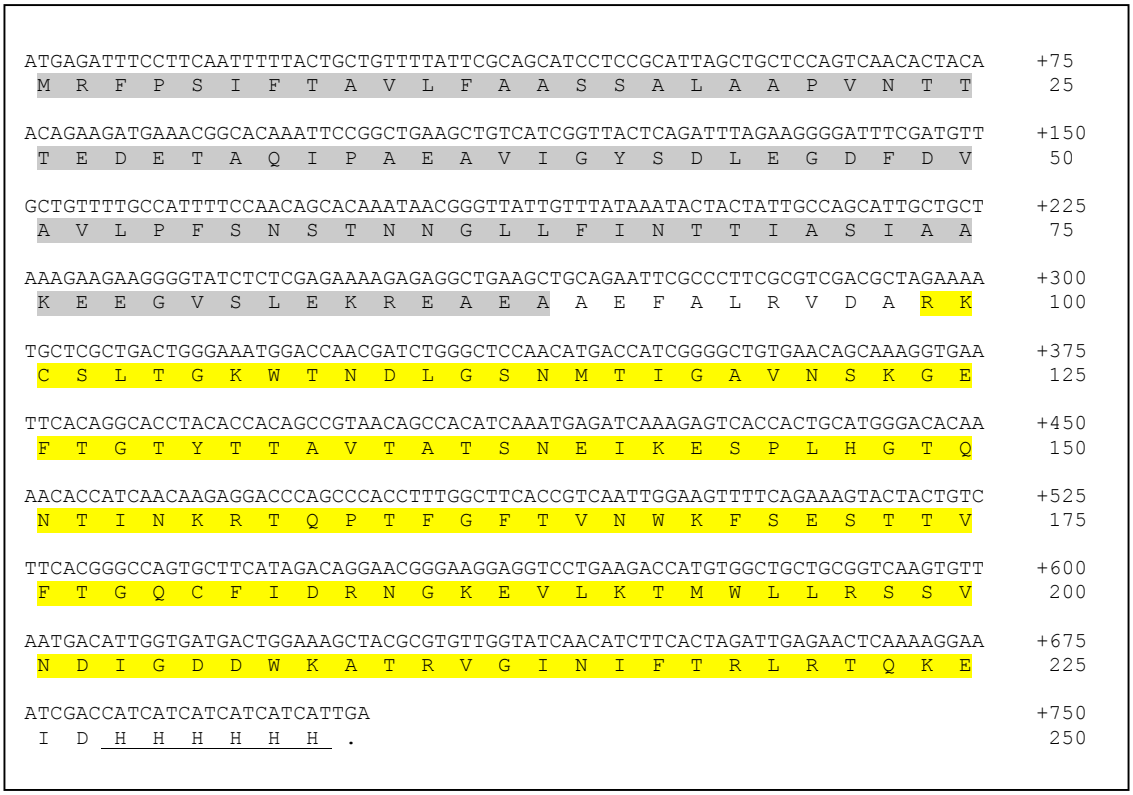
Appendix 1

Nucleotide and Deduced Amino Acid Sequence of Recombinant Avidin



Appendix 1.

Nucleotide sequence and deduced amino acid sequence of recombinant avidin.
The yeast alpha factor sequence of pGAPZαB is highlighted in grey. The avidin sequence is highlighted in yellow. The $(His)_6$ tag is underlined in black.

Appendix 2

Publication:

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Insecticidal activity of recombinant avidin produced in yeast

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ABSTRACT

An expression construct encoding chicken (*Gallus gallus*) avidin was assembled from amplified fragments of genomic DNA. Recombinant, functional avidin was produced in *Pichia pastoris*, with yields of up to 80 mg/l of culture supernatant. The recombinant avidin had similar insecticidal activity to egg white avidin when assayed against larvae of a lepidopteran crop pest, cabbage moth (*Mamestra brassicae*), causing >90% reduction in growth and 100% mortality when fed in optimised diets at levels of 1.5 μM and 15 μM (100 ppm and 1000 ppm wet weight of recombinant protein). The recombinant protein was also highly toxic to a hemipteran pest, the pea aphid (*Acyrthosiphon pisum*), when fed in liquid artificial diet, causing 100% mortality after 4 days when present at concentrations ≥3.8 μM (0.25 mg/ml, 250 ppm). Mortality was dose-dependent, with an estimated LC_{50} of 2.1 μM. Toxicity to *A. pisum* was prevented by biotin supplementation of diet. In contrast, avidin had no significant effects on the survival of cereal aphid (*Sitobion avenae*) at concentrations up to 30 μM in liquid diet. Analysis of genomic DNA showed that symbionts from both aphid species lack the ability to synthesise biotin *de novo*. Cereal aphids appear to be less sensitive to recombinant avidin in the diet through proteolysis of the ingested protein, which would allow recovery of bound biotin.

1. Introduction

Avidin, a protein found in the egg white of birds, reptiles and amphibians, is thought to function as an antibacterial, host-defence protein, by virtue of its ability to bind biotin, an essential vitamin for most organisms. In nature, avidin exists as a homotetramer, comprising four 16 kDa singly glycosylated subunits, each able to bind a biotin molecule with extreme specificity and affinity (Green, 1990).

The search for protein-based approaches to crop protection has been prompted by the growing demand for more environmentally compatible approaches to pest control, and encouraged by the successful use of insecticidal protein toxins from the soil microorganism *Bacillus thuringensis* (*Bt*) as a spray and in transgenic crops. The insecticidal activity of avidin was first identified 50 years ago, when dietary avidin was found to have detrimental effects on the growth of housefly larvae (*Musca vicina*) (Levinson and Bergmann, 1959). Avidin, and the related bacterial protein streptavidin, have since been shown to be toxic to a wide range of insects including representatives of Lepidoptera (Morgan et al., 1993; Du and Nickerson, 1995; Markwick et al., 2001; Burgess et al., 2002; Zhu et al., 2005), Diptera (Levinson and Bergmann, 1959; Tsiropoulos, 1985; Bruins et al., 1991), Coleoptera (Levinson et al., 1967; Allsopp and McGhie, 1996; Kramer et al., 2000; Yoza et al., 2005; Cooper et al., 2006; Murdock and Shade, 2008) and Orthoptera (Christeller et al., 2000). Avidin is a normal component of human diet, and thus has potential for adoption as an insect control agent. Furthermore, dietary levels of the protein required for effective insecticidal activity against a wide range of pests are much lower than levels present in normal human diet. Expression of avidin in transgenic plants has been put forward as a strategy to confer protection against insect pests, with engineering of maize, corn, tobacco, potato, apple and rice having been reported (Kramer et al., 2000; Burgess et al., 2002; Murray et al., 2002; Markwick et al., 2003; Yoza et al., 2005). However, the initial report of expression of avidin in maize, with the intention of producing protein which could be extracted for use as a biochemical reagent (Hood et al., 1997) remains the only example of large-scale expression *in planta*.

The insecticidal effects of avidin are mediated through its biotin-binding activity. In many cases insecticidal effects have been shown to be eliminated in diets supplemented with biotin, supporting the hypothesis that avidin acts through the sequestration of biotin from ingested food, thereby preventing absorption and causing biotin deficiency in the insect. Biotin, as a cofactor of major carboxylases involved in key process such as gluconeogenesis, lipogenesis, and fatty acid and amino acid catabolism is essential for insect growth (Wood and Barden, 1977; Knowles, 1989). Most insects fed on avidin-containing diets show retarded growth, leading to eventual

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mortality. However, susceptibility to the insecticidal effects of the protein shows considerable variability, even between related species (Morgan et al., 1993). This variability in susceptibility is thought to depend upon variations in levels of maternally derived biotin (Markwick et al., 2001).

Most avidin for commercial purposes is purified from hen egg white, although expression systems for producing the protein based on bacteria and insect cells have been described (Airenne et al., 1999). Here, we report the production of recombinant avidin utilising the yeast *Pichia pastoris* as an expression host, which gives a biologically active product at high yield. Bioassays to compare the insecticidal activity of recombinant avidin with native avidin have been conducted, and the potential use of avidin for the control of hempiteran pests is demonstrated. This is the first published report of insecticidal effects of avidin against aphids, important globally as pests of crops not only for the direct damage caused by infestation, but also through their role as vectors of plant diseases.

2. Materials and methods

2.1. Materials and recombinant DNA techniques

All chemical reagents were of GPR or better grade and were supplied by BDH, Sigma or Merck unless otherwise stated. Hen egg white avidin and anti-avidin antibodies were obtained from Sigma. Sub-cloning was carried out using a TOPO-TA cloning kit purchased from Invitrogen. Expression vector pGAPZαB, *P. pastoris* X33 strain, and Easycomp *Pichia* transformation kit were supplied by Invitrogen. Oligonucleotide primers were synthesised by Sigma-Genosys. PCR reactions were performed with Phusion high fidelity DNA polymerase from New England Biolabs following their product recommendations. Plasmid DNA was prepared using the Promega Wizard miniprep kit. Restriction enzymes and DNA ligase were provided by Promega. All general procedures were based on standard protocols described by Sambrook and Russell (2001). DNA sequencing was carried out using Applied Biosystems ABI Prism 3730 automated DNA sequencers by the DNA Sequencing Service (DBS Genomics), School of Biological and Biomedical Sciences, Durham University, UK. Sequence data was analysed using Sequencher software (version 4.5) running on Mac OS computers.

2.2. Electrophoresis and Western blotting

Proteins were routinely separated and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Samples were prepared by adding 5× SDS sample buffer (containing 10% 2-mercaptoethanol) and boiled for 10 min prior to loading. Gels were either stained with Coomassie Blue or transferred to nitrocellulose membrane using an ATTO HorizBlot semi-dry electroblotting system according to manufacturer's instructions. Western blotting was carried out as described previously (Fitches and Gatehouse, 1998), except for chemiluminescent reagents. Enhanced Chemiluminescence detection was carried out using coumaric acid (0.2 mM) and luminol (1.25 mM) in 1 M Tris (pH 8.5) with the addition of 0.009% (v/v) hydrogen peroxide.

2.3. Recombinant avidin preparation

Genomic DNA, used as a template to amplify the avidin coding sequence, was extracted from a 5-day-old chicken (*Gallus gallus*) embryo. The avidin gene contains four exons, with the majority of the mature polypeptide coding sequence present in exons 2 and 3. Exons 2 and 3 were amplified separately using primers; Ex12f with Ex23r (exon 2) and Ex3f with Ex3r (exon 3) (Table 1) and individually sub-cloned into pCR2.1. Positive clones were identified by DNA sequencing. An expression construct for the complete avidin coding sequence (Fig. 1A) was subsequently assembled in a two-step process. In the first step, exons 2 and 3 were assembled together by restricting pCR2.1 clones with *Pst*I and *Sca*I (exon 2) or *Sca*I and *Eco*RI (exon 3) followed by ligation into pUC18 (restricted *Pst*I and *Eco*RI). Again, positive clones were confirmed by DNA sequencing. In the second step, the combined exons 2 and 3 were assembled with annealed oligonucleotides containing the exon 4 sequence (Ex4f and Ex4r; Table 1) and cloned directly into the *P. pastoris* expression vector pGAPZαB. To this end exons 2 and 3 were restricted from pUC18 (*Pst*I and *Mlu*I) and ligated to exon 4 (via *Mlu*I site) and inserted into pGAPZαB (restricted with *Pst*I and *Sal*I). A clone containing the complete expression construct which includes an N-terminal extension of 9 amino acid acids (Fig. 1B) was verified by DNA sequencing, and transformed into the wild-type X33 *P. pastoris* strain using the Invitrogen 'Easycomp' *Pichia* transformation kit in accordance with the manufacturer's instructions. Transformants were selected by plating on media containing zeocin (100 μg/ml). High-expressing clones were identified by western blot analysis of supernatant from small-scale cultures using anti-avidin antibodies (1:5000 dilution).

2.4. Recombinant avidin expression and purification

P. pastoris containing a selected avidin construct was grown in a 7.5 l BioFlo 110 bench-top fermenter (New Brunswick Scientific) and the cells were processed as previously described (Fitches et al., 2004), with one exception: the growing culture was maintained at pH 4.5. Culture supernatant was adjusted and diluted (50:50) with sodium acetate pH 4.0 and sodium chloride to a final concentration of 50 mM and 0.5 M, respectively. Recombinant avidin was purified using nickel affinity chromatography on a HisTrap crude nickel column (GE Healthcare) with a flow rate of 2 ml/min. Bound protein was eluted by the addition of imidazole (300 mM) in 50 mM sodium acetate pH 7.4. Purified avidin was de-salted by dialysis and freeze-dried. The concentration of avidin in lyophilised samples was estimated by comparison with known amounts of standard native avidin (Sigma) on SDS-PAGE gels.

2.5. Gel filtration of recombinant avidin and commercial hen egg white avidin

Analytical gel filtration was carried out on a column of Sephacryl S-200 (GE Healthcare), 1.6 cm diameter × 70 cm, in phosphate-buffered saline (PBS; pH 7.2), at a flow rate of 15 ml/h. The column was calibrated with standard proteins BSA, ovalbumin,

Table 1
Primers and oligonucleotides used to assemble the avidin coding sequence. Enzyme restriction sites are underlined. For Ex4f5′ and Ex4r3′, bases compatible with restriction sites are underlined.

Primer	Sequence	Enzyme site
Ex12f 5′	CGCGTCGACGCTAGAAATGCTCGCTGACTGGGAAATGG	*Sal*I
Ex23r 3′	CGCGAGTACTTTCTGAAAACTTCCAATTGACGGTG	*Sca*I
Ex3f 5′	CGCGAGTACTACTGTCTTCACGGGCCAGTGC	*Sca*I
Ex3r 3′	CGCGACGCGTAGCTTTCCAGTCATCACC	*Mlu*I
Ex4f 5′	CGCGTGTTGGTATCAACATCTTCACTAGATTGAGAACTCAAAAGGAAA	*Mlu*I
Ex4r 3′	ACAACCATAGTTGTAGAAGTGATCTAACTCTTGAGTTTTCCTTTAGCT	*Sal*I

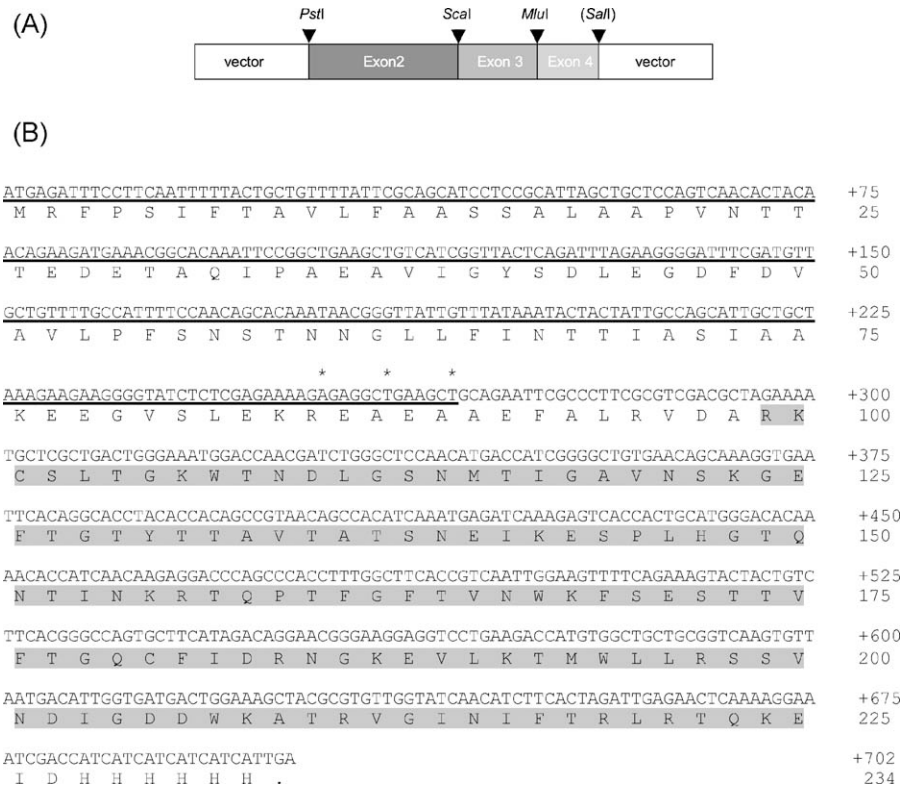

Fig. 1. (A) Diagrammatic representation of avidin expression construct for expression in *P. pastoris* showing restriction sites used for assembly of the gene from fragments amplified from *G. gallus* genomic DNA. (B) Nucleotide and predicted amino acid sequence of avidin expression construct in the vector pGAPZαB. The N-terminal α-factor prepro-sequence (amino acids 1–89) is underlined, predicted Kex2 and Ste13 signal cleavage sites are denoted with * and the mature avidin polypeptide sequence (amino acids 98–227) is highlighted in grey.

and soybean Kunitz trypsin inhibitor, and the elution volume of recombinant and egg white avidin was compared to a standard curve of log (mol. wt.) vs. elution volume.

2.6. Recombinant avidin: in vitro activity

The ability of recombinant avidin to bind biotin was assayed *in vitro* using biotin–agarose (Thermo Scientific). Matrix (1:1 suspension in 100 μl PBS) was incubated with 5 μg of recombinant avidin at room temperature for 30 min. Following centrifugation (1 min at 2000 × *g*) the pellet was washed two times with PBS. Avidin bound to biotin was eluted from the matrix by boiling, and fractions were analysed by SDS-PAGE.

2.7. Recombinant avidin de-glycosylation

Recombinant avidin was de-glycosylated using PNGase F (New England Biolabs), 10 μg of test protein was incubated at 37 °C overnight with 500 units of PNGase F, following the manufacturer's recommended protocol. Yeast invertase (Sigma) was used as a positive control. Reaction products were analysed by SDS-PAGE on 15% acrylamide gels.

2.8. Insects

Insect cultures originally obtained from the Food and Environment Research Agency, York, UK, were subsequently maintained at the University of Durham. *Mamestra brassicae* (cabbage moth) were maintained on artificial diet (Bown et al., 1997) at 23 °C. *Acyrthosiphon pisum* (pea aphid) were reared on established broad bean (*Vicia fabae*) plants at 18 °C, 30% relative humidity. *Sitobion avenae* (cereal aphid) were kept on 4-week old oat (*Avena sativa*) plants at 18 °C, 30% relative humidity. All insects were maintained under a 16 h light:8 h dark regime.

2.9. Lepidopteran bioassay

Purified recombinant avidin was incorporated at known concentrations in artificial diet (Bio-Serv; powder containing cornflour, raw wheat germ, brewers yeast, ascorbic acid, benzoic acid, methyl parabenzoate) to assay for insecticidal activity against newly hatched *M. brassicae* larvae. To prepare diets 1 g diet powder was mixed with 1 ml dist. water. Boiled agar (40 mg in 2 ml dist. water), was added to the mix and the test protein finally added in 1 ml of dist. water. This diet contains biotin as a component of brewers yeast and wheat germ (1 g diet powder contains approximately 0.1 μg biotin; equivalent to approximately 0.02 ppm, based on wet weight, in final diet, or approximately 0.08 μM). Treatments consisted of 1000 ppm, 100 ppm and 10 ppm (based on wet weight) of recombinant avidin re-suspended in water and incorporated into 5 g wet weight of diet. Diets containing egg white avidin (Sigma) and casein at the highest concentration of 1000 ppm were prepared as positive and negative controls, respectively. For each diet concentration, 20 larvae were maintained in clear plastic pots (5 per pot) containing moist filter paper to prevent diet desiccation. Survival was monitored daily and individual weights were recorded daily after 9 days at which time the larvae were large enough to handle without damage.

2.10. Aphid bioassays

Liquid artificial diet (Douglas and Prosser, 1992) was used for the oral delivery of recombinant avidin to *A. pisum* and *S. avenae*. The diet contains biotin at a concentration of 1.0 μg/ml (4.1 μM).

Avidin was incorporated into diet at concentrations ranging from 0.05 to 2.0 mg/ml (0.75–30 μM). Diet-only negative controls (100 μl artificial diet) were also included. A total of 100 μl of solution was used for each aphid feeding chamber, consisting of Perspex rings (40 mm diameter) overlain with 2 layers of stretched parafilm between which the diet was sandwiched. The ability of aphids to survive on diets containing a range of biotin concentrations was assessed by adding biotin at the required concentration to diet prepared without biotin. In addition, a separate assay was conducted where avidin and biotin were included in the diets at concentrations of 0.2 mg/ml (3.0 μM) and 0.1 mg/ml (410 μM), respectively. For assays *A. pisum* and *S. avenae* adult aphids were placed on artificial diet and left to produce nymphs overnight. Adults were then removed and the nymphs were maintained on the diet for a further 24 h. Twenty nymphs per treatment were then transferred to feeding chambers (10 per replicate) containing test diets. Survival of the aphids was monitored daily for 10 days, with the diet refreshed as required. For determination of the effect of avidin on the growth of *S. avenae*, assays were set up as above. After 14 days, aphids were imaged using a Nikon binocular microscope with camera. Captured images were analysed using ImageJ software (NIH; http://rsb.info.nih.gov/ij), measuring overall length and width of aphids.

2.11. Amplification of genomic region containing biotin synthesis genes from Buchnera genomic DNA

DNA was extracted from whole adult aphids using TRI reagent (T9424, Sigma Chemical Company) according to the manufacturer's protocol. Primers for PCR were designed based on the published pea aphid *Buchnera* genome sequence (http://buchnera.gsc.riken.go.jp), using sequences in flanking *glyA* and *ybhE* genes to amplify the region containing genes encoding biotin synthesis enzymes (forward primer 5′-CAAGGTGGACCATTAATGCAT-3′, bases 318,674–318,694: reverse primer 5′-GGTCCGCGTCATATCATCTTTCATCC-3′, bases 323,153–323,128; numbering from accession CP001158). Experiments using *A. pisum* total DNA as a positive control showed that a product of the correct size was amplified; this was cloned into pJET (Clontech) and DNA sequencing was used to confirm that it corresponded to the expected fragment. Amplification of total DNA extracted from *S. avenae* gave a fragment of similar size to that obtained from *A. pisum*. This was cloned as before, and analysed by DNA sequencing, using a "primer walking" method to obtain the complete sequence of the fragment.

2.12. Stability of avidin

Analysis of the stability of recombinant avidin *in vivo* after ingestion by aphids was carried out as follows: 2 day old *A. pisum* and *S. avenae* aphids (20 per treatment) were fed for 48 h on diet containing 2 mg/ml avidin, and for 48 h with diet containing 2 mg/ml avidin followed by control artificial diet for 48 h. Control samples were obtained from aphids fed on artificial diet for 48 h. Aphids were then rapidly frozen in liquid nitrogen and homogenised in 100 μl PBS. Following centrifugation (12,000 × *g*; 2 min), supernatants were analysed for the presence of avidin by western blotting. Mature aphids were starved for 1 h and then fed with control diet and diet containing 2 mg/ml recombinant avidin. Each replicate assay contained 10 aphids contained in a 1.5-ml Eppendorf tube fed on 100 μl of diet. Honeydew was collected from the tubes after 72 h by adding 25 μl SDS-PAGE sample loading buffer to the tubes, and spreading over the surface of the tube. The buffer was collected by brief centrifugation, and the whole sample was loaded into a single well for gel electrophoresis. The proteins present in honeydew were separated by SDS-PAGE.

2.13. Statistical analysis

All data analysis was conducted using the statistical functions of GraphPad Prism 5.0. Kaplan–Meier insect survival curves were compared using Mantel–Cox log-rank tests. Insect weights and sizes were analysed using either Student's *t*-tests or one-way analysis of variance (ANOVA). Analysis of growth curves was carried out by non-linear regression, fitting to a Weibull growth curve model. The accepted level of significance was $P < 0.05$ in all cases.

3. Results

3.1. Expression, purification and characterisation of recombinant avidin

A construct encoding a mature avidin polypeptide (i.e. without the predicted signal peptide) was assembled by PCR using gene specific primers for the three exons of the published sequence of the chicken avidin gene (accession no. AJ311647), and *G. gallus* genomic DNA as a template. The assembled sequence encoded a protein identical to the published sequence for chicken avidin (accession no. CAC34569), with the exception of one amino acid substitution; Asp11 in the database mature protein sequence was replaced by Thr in the assembled sequence. Sequence encoding the mature avidin polypeptide was cloned into the yeast expression vector pGAPZαB, which directs constitutive expression of the recombinant polypeptide. The predicted protein product (Fig. 1B) contains a C-terminal extension coding for a hexahistidine tag, and an N-terminal extension of 9 amino acids derived from the cloning vector, arranged in frame with the N-terminal yeast α-mating factor prepro-sequence present in the expression vector, which directs secretion of the recombinant protein product into the culture medium. The predicted product, after post-translational removal of the α-factor prepro-sequence, contains 144 residues and has a predicted molecular mass of 16.26 kDa. The protein has one predicted glycosylation site (N-X-S/T) located at residue 25. DNA from verified clones was linearised and transformed into the X33 wild-type strain of *P. pastoris*. Clones were selected on zeocin-containing media, and screened for avidin expression by western blotting of culture supernatant.

A high-expressing yeast clone was selected for large-scale culture in a bench-top fermenter. Recombinant avidin was purified from culture supernatant to almost 100% homogeneity by nickel affinity chromatography (Fig. 2A), giving yields of up to 80 mg/l culture supernatant after dialysis and freeze-drying. Purified recombinant avidin runs as a single band of approximately 20 kDa when analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as compared to chicken avidin (commercial) that has an indicated molecular mass of 16 kDa. The recombinant avidin is reactive with anti-avidin antibodies (Fig. 2B) after western blotting, at a level similar to protein purified from egg white. Gel filtration of recombinant avidin and egg white avidin gave identical elution volumes for the two samples within the experimental error of the technique (Fig. 2E), suggesting that the recombinant avidin forms tetrameric molecules similar to "wild-type" avidin, although both samples ran at an indicated molecular weight of approximately 50,000 compared to standard proteins. Recombinant avidin was glycosylated by the yeast expression host, as shown by de-glycosylation *in vitro*. Treatment of the protein using PNGase F resulted in a progressive disappearance of the 20 kDa band seen after SDS-PAGE, and the appearance of a band at 16 kDa (Fig. 2D). Thus the higher molecular weight of recombinant avidin, as compared to native protein, can be attributed to glycosylation by the yeast expression host and also to the presence of N- and C-terminal extensions in the expression construct.

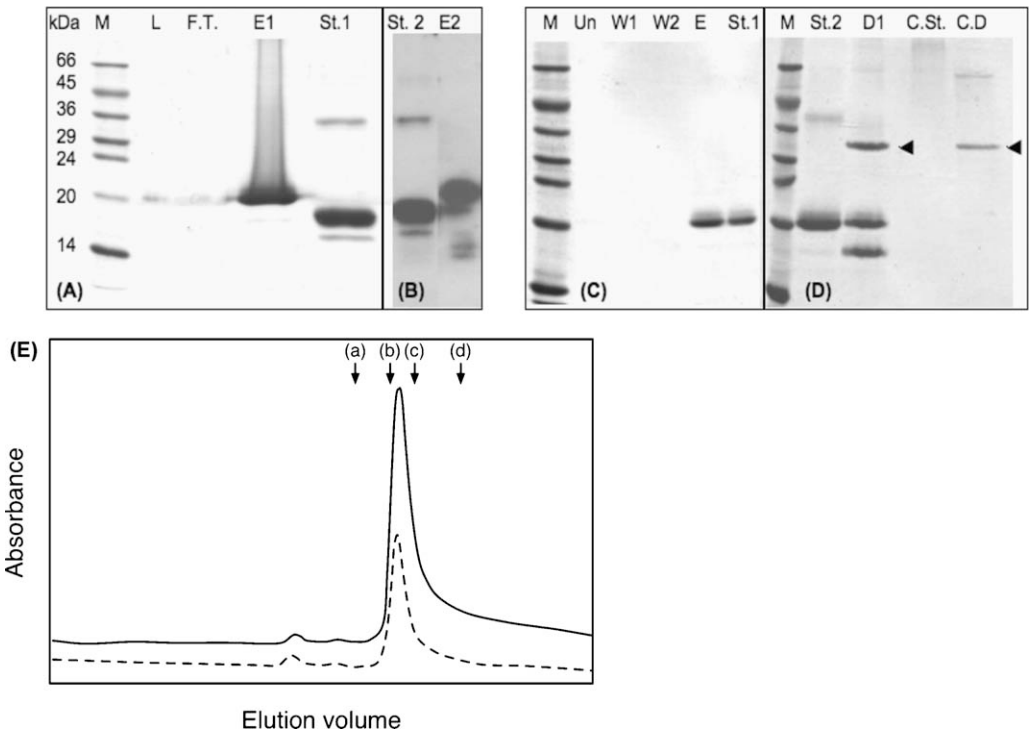

Fig. 2. Characterisation of recombinant avidin. (A) SDS-PAGE of fractions from purification of recombinant avidin from yeast culture supernatant by Ni-NTA affinity chromatography; L, F.T., and E1 denote load, flow through, and elution fractions, St.1 is Sigma avidin (10 μg) M in all cases is standard molecular weight marker SDS-7 (Sigma). (B) Western analysis (anti-avidin antibodies) of purified recombinant avidin (E2, 10 ng), St.2 shows 10 ng Sigma avidin. (C) SDS-PAGE demonstrating *In vitro* binding of recombinant avidin to immobilised biotin–agarose; Un, W1, W2, and E denote unbound, wash, and elution fractions. St.1 is recombinant avidin (2.5 μg). (D) SDS-PAGE following *in vitro* de-glycosylation of recombinant avidin by PNGase F; St.2 and D1 denote avidin and deglycosylated avidin, C.St. and C.D. denote control yeast invertase and de-glycosylated yeast invertase (10 μg loaded in all lanes). Arrows depict PNGase F enzyme. (E) Gel filtration of commercial hen egg white avidin (dashed line) and recombinant avidin (solid line) on Sephacryl S-200 column. Labelled arrow heads show elution volumes of standard proteins: (a) bovine serum albumin dimer (134 kDa); (b) bovine serum albumin (67 kDa); (c) ovalbumin (45 kDa); (d) soya bean Kunitz trypsin inhibitor (20 kDa).

The ability of recombinant avidin to bind to biotin was confirmed *in vitro* by incubation with a molar excess of immobilised biotin. SDS-PAGE analysis (Fig. 2C) showed the presence of avidin in biotin-bound fractions and absence of protein in wash fractions, suggesting that 100% of the purified protein was able to bind to biotin, and that the recombinant protein is fully biologically active.

3.2. Insecticidal activity of orally delivered avidin towards lepidopteran larvae

Neonate larvae of cabbage moth (*M. brassicae)* were fed on artificial diet containing recombinant avidin at concentrations of 1000 ppm, 100 ppm and 10 ppm (wet weight; equivalent to 1.0, 0.1 and 0.01 mg/g diet, or estimated concentrations of 15, 1.5 and 0.15 μM) and egg white avidin at 1000 ppm. As shown in Fig. 3A, recombinant avidin and egg white avidin at 1000 ppm caused a progressive, significant reduction in larval survival with 100% mortality recorded after 21 days and 20 days, respectively. Survival of larvae fed on control diet was ≥90% over this period, which extended from neonate to the final larval instar. The survival curves for the two avidin treatments were similar suggesting that recombinant avidin has a similar insecticidal activity to egg white avidin. Reductions in survival coincided with periods of moults in control larvae suggesting that the limited growth of larvae reduced reserves available for successful ecdysis. Insects fed avidin at 1000 ppm (15 μM) showed severe growth inhibition; after 9 days exposure (first point at which insects could be individually weighed) experimental insects were approximately 70% of control weight. Control insects increased their mean weight by nearly 18-fold over the remainder of the experiment (12.3 mg on day 9 to 216 mg by day 21), with a steady increase in mean weight over time, whereas surviving avidin-fed insects at most doubled their weight, and showed no consistent trend of weight increase. The effects of recombinant and egg white avidins at 1000 ppm (15 μM) on larval growth were similar.

The insecticidal effects shown by recombinant avidin were dose-dependent (Fig. 3B). Feeding recombinant avidin at concentrations of 1000 and 100 ppm (15 μM and 1.5 μM) both resulted in 100% larval mortality over 21 days, with similar survival curves. On the other hand, feeding recombinant avidin at a concentration of 10 ppm (0.15 μM) had no effect on survival, with 90% survival over 21 days, and a survival curve similar to control insects. The LD_{50} for recombinant avidin in these assays can thus be estimated as being approximately 30 ppm (approximately 0.5 μM). Effects of 1000 ppm and 100 ppm (15 μM and 1.5 μM) avidin treatments on larval growth were similar, with very strong inhibitory effects on surviving larvae observed (approximately 90% reduction in mean weight compared to control to day 20). On the other hand, the 10 ppm (0.15 μM) avidin treatment had only a marginal effect on growth, with no effect compared to controls over the first 13 days of the assay, and approximately 15% reduction in growth over remaining period of the assay, with a final difference in mean larval weights of approximately 20%. The reduction in growth was statistically significant when data were analysed using a Weibull growth curve model ($P < 0.02$, null hypothesis that one curve fits all data).

3.3. Insecticidal activity of orally delivered avidin towards aphids

The oral activity of avidin towards two hemipteran pest species was evaluated by feeding pea aphids (*A. pisum*), and cereal aphids

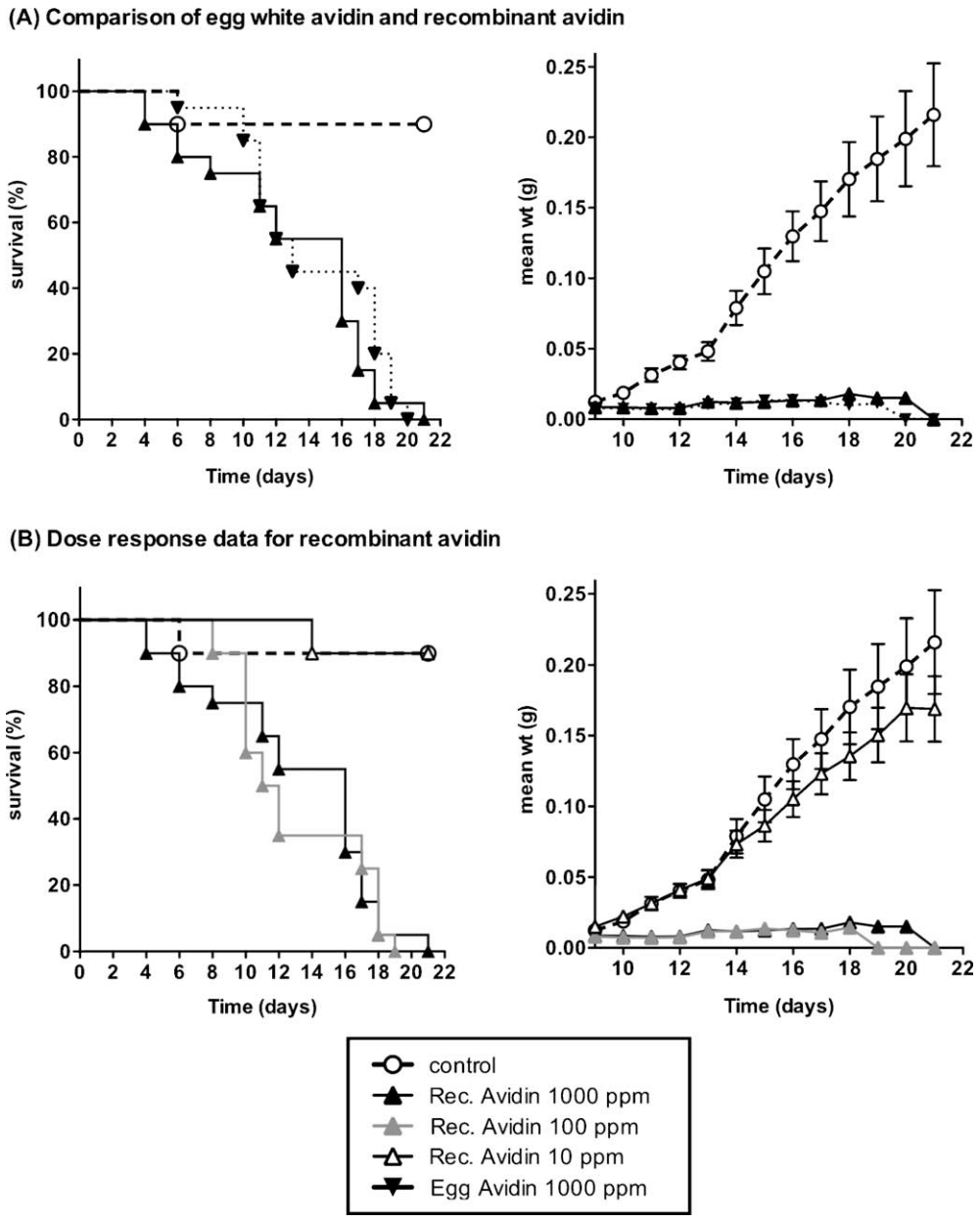

Fig. 3. Effects of avidin on survival and development of cabbage moth (*M. brassicae*) neonate larvae. (A) Comparison of egg white avidin and recombinant avidin incorporated into diets at 1000 ppm (wet weight). Larval weights were recorded from day 9 onwards when the insects were large enough to handle without damage. Points on weight graph show mean ± S.E. (B) Dose–response effects of recombinant avidin incorporated into diets at levels 10–1000 ppm (wet weight). Points on weight graph show mean ± S.E.

(*S. avenae*) on artificial diets containing recombinant avidin. Concentrations of avidin in the range 0.05–2.0 mg/ml (0.75–30 μM, 50–2000 ppm) were employed. The standard aphid diet contained a background level of biotin of 4.1 μM.

Recombinant avidin had a dose-dependent insecticidal activity towards *A. pisum*, causing a reduction in aphid survival (Fig. 4). Acute toxicity was observed in diets containing 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml avidin with 100% mortality (Mantel–Cox tests, $P < 0.0001$) recorded for all treatments 4 days after the onset of the assay. Aphid survival was also reduced in treatments containing 0.2 mg/ml, 0.1 mg/ml, and 0.05 mg/ml avidin with respective values of 20%, 70%, and 80% recorded at the end of the 10-day assay, as compared to 95% control survival. Differences in survival as compared to controls were significant for the 0.2 mg/ml treatment (Mantel–Cox, $P < 0.0001$) but not for the 0.1 mg/ml and 0.05 mg/ml treatments. At sub-lethal levels of avidin, mortality was observed in the first 6 days of the assay, after which no further drop in survival was recorded. Control survival was greater than 90% over the assay period. Fitting data to a dose–response curve equation allowed an LC_{50} of 0.14 mg/ml (2.1 μM) to be estimated for avidin toxicity to pea aphid (Fig. 4B); similar values were obtained whether survival at day 4 or day 10 of the assay was used to estimate LC_{50}. Supplementation of diet with biotin prevented the insecticidal effects of avidin; pea aphids fed diets containing avidin and biotin (at concentrations of 2.0 mg/ml and 0.1 mg/ml; 3.0 μM and 410 μM, respectively) showed no reduction in survival compared to controls (results not shown). This demonstrated that the insecticidal activity of recombinant avidin towards pea aphids was attributable to biotin deficiency caused by the sequestering of biotin by avidin.

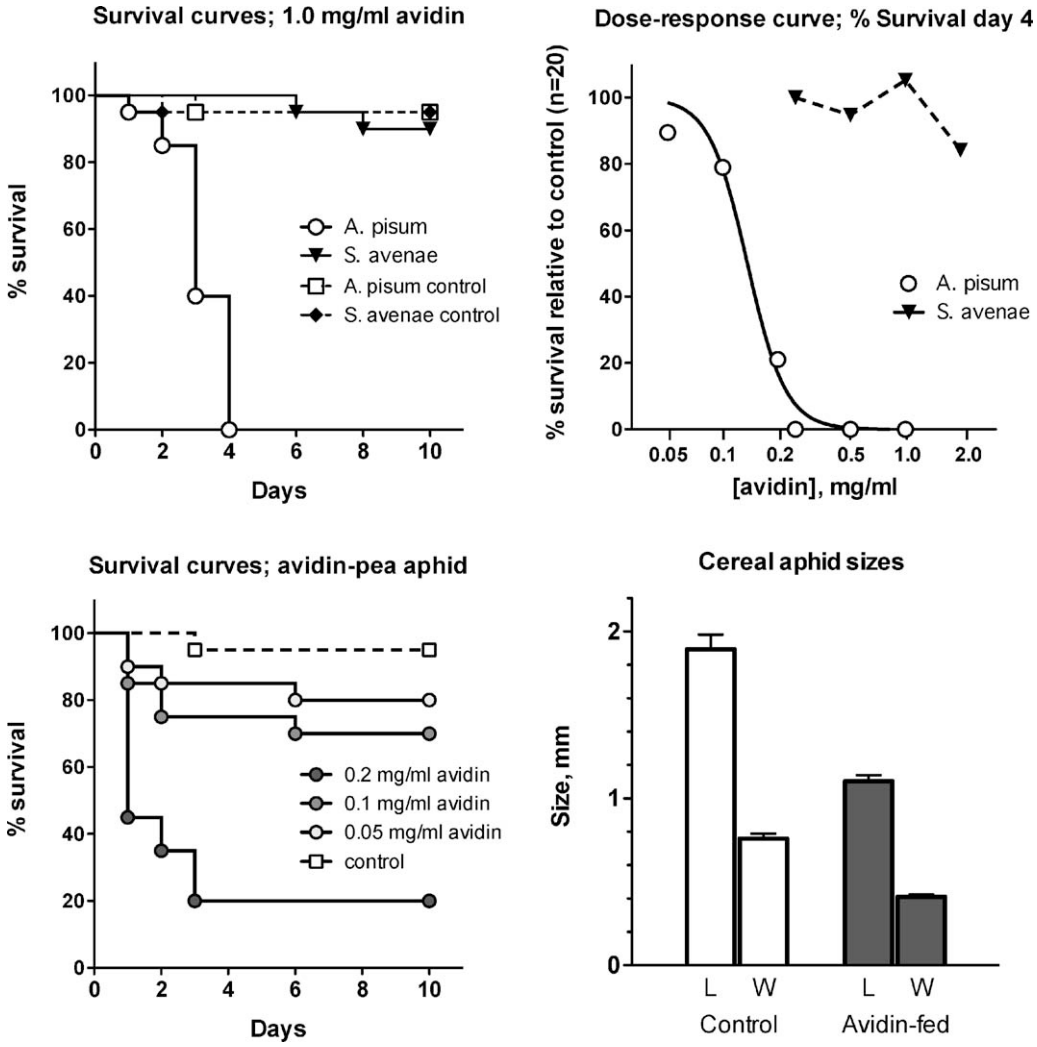

Fig. 4. Survival of pea aphids (*A. pisum*) and cereal aphids (*S. avenae*) aphids fed on artificial diet containing recombinant avidin. (A) Comparison of effects of avidin at 1.0 mg/ml on survival of pea aphid and cereal aphid. Survival of pea and cereal aphids on control treatments was identical (symbols overlap on graph). (B) Dose–response curves for survival at day 4 of assay. Data for pea aphid are fitted to a standard dose–response curve using non-linear regression. Data for cereal aphid did not fit to a curve due to low mortality at all doses. (C) Survival curves for pea aphids fed doses of avidin lower than estimated LC_{50}. (D) Effect of avidin on growth of cereal aphids. Aphids were fed from neonate on diet with and without added avidin (2 mg/ml); measurements taken after 14 days show mean length (L) and width (W) ± S.E. (n = 10 per treatment).

In contrast to effects on *A. pisum*, when recombinant avidin was fed to *S. avenae* no significant effects on aphid survival at concentrations up to 1.0 mg/ml (15 μM; Fig. 4A and B) were observed. Even when avidin was added to diet at a concentration of 2.0 mg/ml (30 μM) aphid survival after 10 days of feeding was only reduced to 75%, compared to 95% for control fed insects (Fig. 4B). However, avidin did have an effect on growth of *S. avenae*, since all of the aphids exposed to avidin-containing diets (concentration range 0.25–2.0 mg/ml, 3.8–30 μM) were observed to be smaller than the control aphids after the initial 3–4 days of the assay, and during the remainder of the assay period. Measurements carried out on *S. avenae* fed avidin at 2.0 mg/ml (30 μM) showed that after 14 days exposure to avidin-containing diet, aphids had only grown to approximately 60% of the size of controls (Fig. 4D).

3.4. Mechanism of toxicity of avidin towards aphids

When *A. pisum* and *S. avenae* were fed on diets containing varying amounts of biotin, from 0 to 4 μM (normal diet level), mortality was observed in both species at biotin levels ≤3 μM, but the two species showed differing responses to sub-optimal concentrations of biotin (Fig. 5A). Pea aphids fed control diet containing 4 μM biotin grew normally with no mortality, whereas aphids fed levels of biotin ≤3 μM showed similar mortality to aphids fed no biotin at all, with 100% mortality at day 9 for all treatments. Survival curves for *A. pisum* fed biotin in the range 0–3 μM were not significantly different. *S. avenae* were less sensitive to sub-optimal biotin levels in diet, with 100% mortality at day 9 and similar survival curves for treatments in the range 0–2 μM, but only partial mortality (55%) for the 3 μM biotin treatment (0% mortality for 4 μM biotin control). The survival curve for the 3 μM biotin treatment was significantly different from both the "normal" diet control and the 0–2 μM biotin treatment curves.

The two aphid species also showed differing responses when fed diet containing no biotin, or a sub-lethal level of avidin (0.2 mg/ml for *A. pisum*, 2 mg/ml for *S. avenae*; Fig. 5B). In this assay, survival curves for pea aphid on biotin-free diet ± avidin were not significantly different, with 100% mortality at day 7 (without added avidin) or day 9 (with added avidin). In contrast, addition of avidin to the *S. avenae* diet improved survival significantly; 100% mortality was observed at day 10 for aphids on diet with no added avidin, but 75% of aphids on diet containing 2 mg/ml avidin survived to day 10. Survival curves for these two treatments were significantly different.

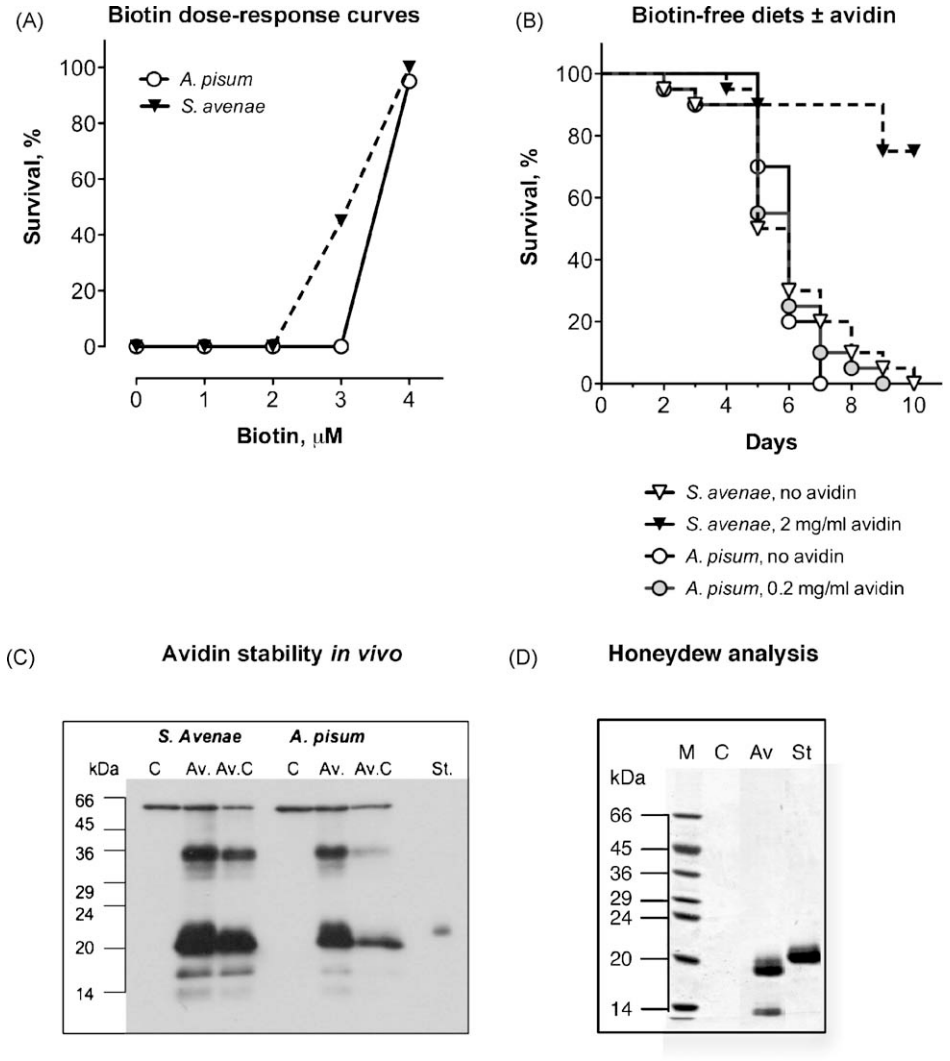

Fig. 5. Mechanism of avidin toxicity towards aphids. (A) Survival of aphids fed on diet containing varying levels of biotin. Survival at day 9 (100% mortality of aphids on biotin-free diet) is plotted against concentration of biotin in diet. (B) Survival of aphids on biotin-free diets containing sub-lethal levels of avidin. Survival curves for different treatments as described in legend. (C) Western analysis (anti-avidin antibodies) of whole *S. avenae* and *A. pisum* protein extracts prepared directly after feeding on recombinant avidin for 24 h (Av.), or after chasing avidin with control diet for 48 h (Av.C). C denotes samples from control-fed aphids. St. is 10 ng standard recombinant avidin, the position of standard protein marker mix (SDS-7, Sigma) run on the blotted gel is depicted. (D) Proteins present in honeydew from aphids feeding on control diet and diet containing recombinant avidin; analysis of honeydew by SDS-PAGE. Lanes are taken from a single gel; omitted tracks contain replicates. C denotes honeydew from control diet-fed aphids, Av honeydew from aphids fed on avidin-containing diet. St is recombinant avidin standard (2 μg) and M is the molecular weight marker.

Supplying recombinant avidin to *S. avenae* therefore compensated almost wholly for a lack of biotin.

To investigate the possibility that the *Buchnera* symbionts in *S. avenae* contained a functional operon for biotin synthesis, the sequence of the region of *Buchnera* genome containing the biotin operon was determined. A fragment of approximately 4.5 kbp was amplified from DNA isolated from *S. avenae*, using primers based on the published *A. pisum Buchnera* genome sequence. This fragment was fully sequenced (Fig. 6). The determined sequence was very similar to the published genome sequence for *Buchnera aphidicola* from *A. pisum*, containing parts of the *glyA* and *ybhE* genes which flank the *bio* operon (Fig. 6), and open reading frames for the polypeptides encoded by the *bioA*, *bioB* and *bioD* genes (data not presented). This result shows that the *S. avenae* symbionts, like

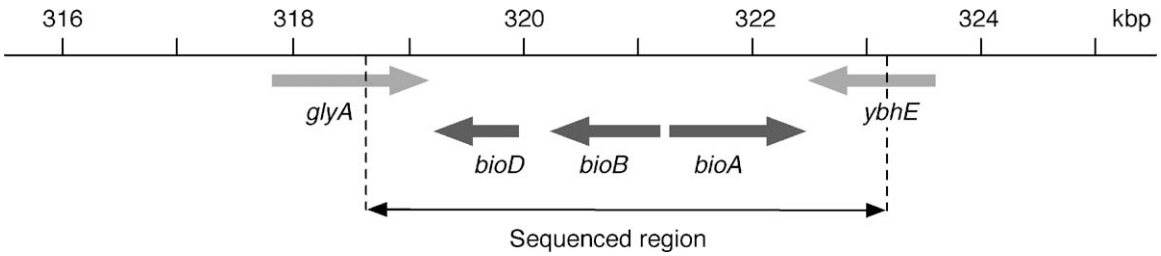

Fig. 6. Map of region of genome (from *Buchnera aphidicola*; accession CP001158) sequenced in *Buchnera* from *S. avenae*. Genes flanking the *bio* operon are shown in light grey, *bio* operon genes in dark grey.

A. pisum symbionts, lack the full complement of genes necessary for biotin synthesis.

To determine whether differences in the insecticidal activity of avidin towards the two aphid species were attributable to differences in the susceptibility of the protein to proteolytic degradation *in vivo*, whole insect protein extracts from aphids fed avidin-containing diet were analysed by western blotting. Samples were prepared directly from aphids fed diets containing avidin at 2 mg/ml (30 μM) for 48 h, and also from aphids that had been chase-fed with control diet. As shown in Fig. 5C, avidin was detected in extracts from both aphid species. Although levels of avidin detected in extracts from aphids that had been "chased" with control diet were lower than in aphids without the chase, the protein was still present in readily detectable amounts, suggesting that ingested avidin had bound to the gut or had been sequestered, and that not all ingested protein had been excreted in honeydew. No avidin was detected in aphids fed control diet. The major band of approximately 20 kDa in extracts of *A. pisum* and *S. avenae* detected by anti-avidin antibodies corresponds to the molecular mass of recombinant avidin. Immunoreactivity of a higher molecular weight protein (approximately 36 kDa) is thought to represent the presence of dimeric avidin. A polypeptide of approximately 16 kDa, also immunoreactive with anti-avidin antibodies, is present in *S. avenae* but only at very much lower levels in *A. pisum*. This suggests that avidin may be more subject to proteolytic degradation following ingestion by *S. avenae* than in *A. pisum*, although most of the protein present in the insect remains largely intact in both species. To confirm that *S. avenae* is able to carry out proteolysis on ingested avidin, honeydew produced by cereal aphids feeding on avidin-containing diet was analysed. As shown in Fig. 5D, excreted avidin showed evidence of proteolytic degradation, with a prominent fragment band at approximately 14 kDa.

4. Discussion

Avidin is a commonly used tool in numerous biological applications, collectively known as (strept)avidin–biotin technology. Bacterial and eukaryotic expression systems have been developed for the production of recombinant avidin, although relatively poor yields (5–10 mg/l culture) have been reported (reviewed by Laitinen et al., 2006). Poor yields are often encountered due to detrimental effects of biotin sequestration by expressed avidin on cell growth. The most successful system for producing recombinant avidin has been transgenic maize, where yields of 150–300 mg avidin were obtained per kg of seed (Hood et al., 1997); the product is commercially available (e.g. Sigma–Aldrich A8706), although most applications continue to use avidin purified from hen egg white.

The *P. pastoris* expression system described in this paper yields 80–100 mg avidin/l culture supernatant, through the use of a secreting system based on the yeast α-factor prepro-sequence to overcome negative effects of biotin sequestration in cells. Despite a constitutive promoter being used to direct avidin expression, cultures grew to high cell densities, and high levels of recombinant protein were produced. In a previous report, an inducible promoter system (AOX) was used to express a modified avidin in *P. pastoris* (Zocchi et al., 2003). Yields of 330 mg/l of avidin after methanol feeding were reported. The use of the constitutive GAPDH promoter in the present work avoids the use of potentially hazardous feedstocks such as methanol, at the cost of a lower yield of recombinant protein. In both cases, *P. pastoris* offers an attractive expression system for efficient production of functional recombinant avidin.

The recombinant avidin produced in *P. pastoris* forms tetrameric molecules similar to egg white avidin, and is fully functional in terms of biotin binding, although a binding constant for the interaction was not measured. The indicated molecular weight on SDS-PAGE of recombinant avidin is significantly greater than egg white avidin, to a greater extent than would be accounted for by the N-terminal extension of 9 amino acids and the hexahistidine tag sequence added to the C-terminus. The single N-glycosylation site in avidin is utilised in both egg white and recombinant avidin. *P. pastoris* carries out core glycosylation plus the addition of mannose residues, normally adding approximately 2 kDa to the molecular weight of a polypeptide (Cregg et al., 1993). In agreement with the present results, glycosylation of recombinant avidin expressed in *P. pastoris* has been reported previously (Schenk et al., 2008). De-glycosylation of recombinant avidin gives a polypeptide whose estimated molecular weight is in agreement with the predicted value. Glycosylation of egg white avidin (Green, 1990) differs from that observed in recombinant avidin expressed in *P. pastoris*, but the carbohydrate side chain does not affect the functional properties of the protein (Nardone et al., 1998).

Much research has been conducted to explore the potential use of avidin as an insect control agent, and early reports of avidin toxicity (Morgan et al., 1993; Du and Nickerson, 1995) suggest that avidin is generally toxic to lepidopteran larvae. The present results showing toxicity towards *M. brassicae* larvae are consistent with previously published data. When incorporated into artificial diet at 100 μg/ml (100 ppm; 0.15 μM), avidin was found to cause greater than 90% mortality of lepidopteran light brown apple moth *Epiphyas postivittana* and green-headed leaf-roller *Ctenopseustis obliquana* after 28 days of feeding (Markwick et al., 2001). Burgess et al. (2002) reported 100% mortality of *Helicoverpa armigera* and *Spodoptera litura* larvae after feeding for 22 and 25 days respectively, on transgenic tobacco expressing 3.1–17.3 μM avidin, and transgenic tobacco and apple plants expressing avidin at 3.1–4.6 μM and 1.9–11.2 μM were resistant to larvae of potato tuber moth (*Phthorimaea operculella*) and *E. postvittana* respectively, with >90% mortality observed on tobacco after 9 days, and 80–90% mortality on apple after 21 days (Markwick et al., 2003). The dose response observed in the present assays, with no effect on survival produced by avidin at 10 ppm (0.15 μM), compared to 100% mortality at 100 ppm and 1000 ppm (1.5 μM and 15 μM), is also in agreement with previous studies which have shown a threshold effect, whereby avidin concentrations above a certain level cause significant mortality, and those below do not (Morgan et al., 1993; Markwick et al., 2001; Burgess et al., 2002; Zhu et al., 2005).

While toxicity of avidin to insects from a range of orders has been demonstrated, no previous studies have reported effects on hemipteran insects. Here we report that avidin shows dose-dependent toxicity to *A. pisum*. The dose–response curve for avidin in these assays shows a sharp transition between no significant effect on survival, and complete mortality, similar to the threshold effect described previously, although an LC_{50} value of 2.1 μM could be estimated. This value is comparable to the level of biotin in the standard diet (4.1 μM; one molecule of avidin binds 4 molecules of biotin, and thus the LC_{50} is a 2-fold molar excess of avidin binding sites over biotin), and suggests that these insects are highly biotin-dependent. In contrast, avidin had no significant effects on the survival of cereal aphids (*S. avenae*) even when incorporated at 2.0 mg/ml (2000 ppm; 30 μM) in artificial diet. Both insect cultures were maintained on plants, and thus have a similar biotin "background".

Reports of immunity to the effects of avidin are rare. Kramer et al. (2000) reported that of 11 coleopteran species tested only one, the larger grain borer (*Prostephanus truncates*), exhibited a tolerance for avidin, where 17% mortality was recorded at a dietary level of 1000 ppm. Malone et al. (2002) reported that whilst avidin had detrimental effects on larvae of clover root weevil (*Sitona lepidus*) it was ineffective against adults; similarly,

Markwick et al. (2001) found that avidin had limited toxicity to larvae of green-headed leaf-roller (*Planotortrix octo*) at concentrations up to 100 μg/ml (100 ppm; 0.15 μM). The failure of avidin to show insecticidal effects on some species has been ascribed to availability of biotin sequestered into eggs and/or embryos by parental generations, a view supported by experiments in which the LC_{50} of avidin towards *E. postvittana* larvae was decreased over 10-fold as a result of feeding for 5 generations on biotin-free diet (Markwick et al., 2001).

The lack of toxicity of avidin towards *S. avenae* cannot be due to a lack of dependence on exogenous biotin, because these aphids do not survive on diets containing no biotin, or low levels of biotin, although they appear to be slightly less sensitive than *A. pisum* to sub-optimal biotin concentrations. The symbiotic *Buchnera* bacteria in aphids have varying complements of the genes involved in the biotin biosynthesis pathway, depending on aphid host species (BuchneraBase; http://www.buchnera.org), but *B. aphidicola* APS in *A. pisum* has lost the capacity to synthesise biotin, having only 3 of the 5 genes involved in biotin biosynthesis (KEGG database; http://www.genome.jp/kegg/kegg2.html). The requirement of *A. pisum* for dietary biotin is thus not surprising. Based on the assays carried out, the *Buchnera* symbiont in *S. avenae* (genome not available) cannot complement a biotin deficiency either. Amplification of the biotin synthesis operon from *S. avenae* provided confirmation that the *Buchnera* symbiont in this aphid species lacks the full complement genes necessary for "*de novo*" synthesis of biotin.

Another possible explanation for the lack of toxicity of avidin towards *S. avenae* compared to *A. pisum* might be differences in avidin interactions with the gut surface. However, in both aphids the protein is retained in the gut, and is detected in the insect even after a chase with non-avidin-containing diet, showing that rapid elimination of avidin, which might prevent sequestration of endogenous biotin, does not occur in cereal aphids. The most plausible explanation for the lack of toxicity of avidin towards *S. avenae* is that biotin bound to avidin, which is not available as a metabolic cofactor, can be recovered from the protein. The ability of *S. avenae*, but not *A. pisum*, to survive on biotin-free diets supplemented with avidin is direct evidence to support biotin scavenging in *S. avenae*. Recombinant avidin contains biotin, as the culture medium in which the yeast is grown is supplemented with this vitamin. The biotin-binding sites on recombinant avidin cannot be saturated, since the protein binds to immobilised biotin, but since tetrameric molecules are formed (Fig. 2E), if a proportion of binding sites contain biotin the protein will still show functional activity and contain biotin. The strength of the biotin–avidin interaction means that the only feasible mechanisms for biotin scavenging are either denaturation of avidin, which is not likely under conditions in the aphid gut, or proteolytic degradation. Evidence for degradation of avidin by *S. avenae in vivo* has been presented (Fig. 5C and D). Although protein digestion in the guts of aphids has been considered unlikely, more recent results have shown that cotton-melon aphid (*Aphis gossypii*), contains cathepsin L-like cysteine proteinase activity in the gut (Deraison et al., 2004), and have identified a gut-expressed family of cathepsin B-like proteases in *A. pisum* (Rispe et al., 2008). In the present case, the data suggest that a combination of lower sensitivity to sub-optimal levels of biotin, and a more effective system for scavenging biotin from avidin in the diet, makes *S. avenae* relatively insensitive to the insecticidal effects of this protein when compared to *A. pisum*. In support of this conclusion, measurements of protein digestion in gut extracts from *S. avenae* and *A. pisum*, using a labelled protein substrate, have shown that the cereal aphid has approximately twice as much gut proteolytic activity as pea aphid on a per aphid basis (data not presented).

Avidin may have potential to control some homopteran plant pests, as well as other insects, if expressed in transgenic plants so that it is available to phloem feeders. Further investigation of its insecticidal activity is warranted, especially in view of the significance of homopteran spp. as global plant pests (not only through direct damage to plants, but also as vectors of plant disease) for which no *Bt*-based crop protection is currently available.

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